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ELABORATO DI LAUREA

Characterization, maintenance, and application of Bottlenose dolphin (*Tursiops truncatus*) cell lines: an *in vitro* model for cold plasma treatment

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1. ABSTRACT

Bottlenose dolphin is a protected species and the opportunities for tissues sampling from freshly dead animals suitable for cell culture are rare.

For this reason, the establishment of new *in vitro* cell lines represent new opportunities for acquiring knowledge on physiological and pathological aspects of this marine mammals.

In this thesis, the first goal was to set up a protocol to maintain in culture a skin-derived cell line and a brain-derived cell line obtained from the cetacean Bottlenose dolphin (*Tursiops truncatus*)

The skin and brain-derived cell lines were cultured and maintained in standard conditions (at 37°C and 5% CO_2) in a cell culture medium consisting of DMEM-F12 supplemented with 10% of fetal bovine serum (FBS). The cells were morphologically analysed by haematoxylin and eosin staining and the ultrastructure of cells was examined by Transmission electron microscopy observation (TEM). Moreover, cells were characterized by immunocytochemical analysis. The cells of brain derived cell line were immunoreactive to the vimentin and ki-67 markers.

The second goal of this thesis focused on investigating the effects of increasing exposure time to cold atmospheric plasma (CAP) in the cells of the Bottlenose dolphin skin-derived cell line. The cell viability was evaluated through the MTT assay, after different exposure time to cold plasma treatment (1, 2, 5, 6, 7, 8 9 and 10 minutes respectively), assessing the effects after two incubation time: after 0 hours and after 8 hours, from the treatments.

Through a high-throughput imaging analysis, we screened and quantified cell nuclei in the diverse phases of the mitotic cell cycle or involved in process of cell death. Our preliminary results showed an inhibition of cell proliferation and an increasing of cell death after long-time cold plasma treatment in comparison to short-time treatment in Bottlenose dolphin skin-derived cells.

In this study, we showed the potential of *in vitro* model obtained from marine mammals, focusing on its versatility and possible applications for testing the effects of novel biomedical technique as the CAP treatment in alive cells.

1. INTRODUCTION

1.1 CELL LINES OBTAINED FROM MARINE MAMMALS

1.1.1 The Bottlenose dolphin (*Tursiops truncatus*)

The well-known and extensively researched Bottlenose dolphin is a cetacean species that inhabits temperate and tropical waters. They live in a variety of environments, such as shallow coastal waters, bays, lagoons, and estuary systems (Baird et al., 2009). There are two known ecotypes of bottlenose dolphins: the shallow, nearshore species found in warm waters, and the deep, offshore species found in colder waters (Duffield, Ridgway, and Cornell, 1983; Fruet et al., 2017; Perrin, Thieleking, Walker, Archer, and Robertson, 2011; Segura, Rocha-Olivares, Flores-Ramírez, and Rojas-Bracho, 2006). Males can live up to 48 years, and females can live up to 57 years (Wells and Scott, 1999). These are long-lived mammals. Different populations, age groups, and sexes have different diets (Barros, Parsons, and Jefferson, 2000; Blanco, Salomón, and Raga, 2001; Gannon and Waples, 2004; Giménez et al., 2017; Santos, Fernández, López, Martínez, and Pierce, 2007). The Bottlenose dolphin is protected as Vulnerable in the Mediterranean subpopulation (Bearzi, Fortuna, and Reeves, 2012), Least Concern on the International Union for Conservation of Nature's (IUCN) Red List of Endangered Species (Wells, Natoli, and Braulik, 2019). It is listed in Appendix II of CITES.

1.1.2 The relevance of cell cultures as *in vitro* model

Cetaceans are considered sentinel animals because they live long lives, live near coasts, have high trophic levels, and have substantial blubber reserves. (Bossart, 2011). Early environmental responses are facilitated by these systems (Bossart, 2011; Reddy, Dierauf, and Gulland, 2001). However, in vivo studies on cetaceans are limited because of their protected status (Hunt et al., 203). The biological material and tissue samples that stranded cetaceans provide shed light on the potential and state of research. (Peltier et al., 2012, 2014). Today, research on living cetaceans is mainly limited to the collection of biological samples using minimum invasive and non-invasive techniques, such as fecal, blow and blubber samples, skin biopsies and photographic data (Hunt et al., 2013). However, collecting data in their natural environment if often very difficult and it usually requires very specific and generally expensive logistics. Another valuable source of biological material from free-ranging cetacean populations are deceased animals. These samples can be used as living cell cultures and can reveal details on their biology, genetics, anatomy, physiology, and disease. (Ballarin et al., 2005).

1.2 THE COLD ATMOSPHERIC PLASMA (CAP)

The ionized gas known as non-thermal atmospheric pressure plasma (NTAPP) is made up of photons, free radicals, and charged particles. Biological effects of NTAPP radiation on cells, both direct and indirect, include cell death and wound healing. Applying a radiofrequency (RF) electric field to a stream of helium flow at atmospheric pressure resulted in the plasma. Plasma was generated between two grids acting as electrodes, and the cultured cells were exposed to the so-called afterglow, which is the chemical-enriched helium flow. In contrast to other cold plasma devices used in biomedical applications, the plasma- induced charged species did not have a direct biological effect on the cultured cells since they recombined very quickly before reaching the samples. (Martines et al., 2009)

The generation of reactive oxygen species (ROS) is stimulated by the interaction between the electron population and the surrounding air mixed with the helium flow (Valko et al., 2007). ROS have long been thought to be unintentional consequences of oxygen metabolism that are harmful to cell viability. However, more recent studies have revealed the dual nature of ROS and their significance in maintaining regular biological processes (Rhee et al., 2006). Elevated reactive oxygen species levels have been linked to ageing, cancer, and neurological illnesses. On the one hand, they cause permanent damage to cellular organelles, membranes, proteins, and DNA (Brun et al. 2012).

The cold plasma treatments have been tested in mammalian species and in human cell lines (Brun et al. 2014) and the innovation of this study is based on the application of cold plasma treatment on the bottlenose dolphin skin-derived cell line.

1.3 AIMS OF THE STUDY

In vivo investigations in protected cetacean species are nearly impossible to conduct. Therefore, the development of new cetacean cell lines, allow to investigate physiological and toxicological responses in living cells in a standardized model.

The aims of the present thesis are:

- to maintain and culture and grow two cell lines obtained from the marine mammal Bottlenose dolphin (*Tursiops truncatus),* in particular a skin-derived cell line and a brain-derived cell line.
- to characterize the brain-derived cell line, including shape analysis and morphology by haematoxylin and eosin staining, ultrastructural analysis and immunocytochemistry characterization.
- To evaluate the effects of increasing time-exposure of cold atmospheric plasma treatment in Bottlenose dolphin skin derived cells.
- The cell viability was evaluated through the MTT assay and by cell cycle analysis, by high-throughput screening analysis, after different exposure-times and incubation-times from the treatments.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagent preparation

Cell culture medium

- DMEM and Ham's F-12 (Biowest®) 80-90 % (v/v)
- FBS Good (Pan Biotech™) 10-20 % (v/v)
- Antibiotics mixture of penicillin (30 mg l^{-1}), streptomycin (50 mg l^{-1}) (Pan Biotech™) 1% (v/v)
- Essential Amino Acids 1% (v/v)

Cell cryopreservation medium

- FBS Good (Pan Biotech™) 90% (v/v)
- DMSO (Sigma-Aldrich) 10% (v/v)

PBS 10X pH 6.8

- 500 ml Milli-Q® Water
- NaCl: 43,8 gr
- NaH2PO4 (dibasic anhydrous): 0,78 gr
- NaHPO4 6,17 gr

* PBS 1X is obtained by preparing a solution containing 10% PBS 10X and 90% Milli- Q® Water (v/v), pH 7.4

2.2 METHODS

2.2.1 Maintenance and growing of two cell lines obtained from diverse tissues of Bottlenose dolphin *(Tursiops truncatus)*

The immortalized Bottlenose dolphin brain-derived cell line and skin-derived cell line were previously established and characterized following an established laboratory protocol (Suman et al., 2012; Otero-Sabio et al., 2022). The cell lines were cultured and maintained in a medium consisting of a 1:1 mixture of DMEM and Ham's F-12 (Biowest®), supplemented with penicillin (30 mg l^{-1}), streptomycin (50 mg l^{-1}) (Pan Biotech) and 10% fetal bovine serum

(FBS Good, Pan Biotech™). Cells were maintained in an incubator under standard conditions at 37 ◦C with 5% CO_2 and humidified atmosphere.

At 80% cell confluence, cell culture medium was discarded, and cells were washed with warm sterile PBS. Then, cells were than incubated with trypsin-EDTA (Biowest®) at 37 °C until they detached from the bottom of the flask (2 min). The enzymatic digestion of trypsin was stopped by adding 3 ml of fresh cell culture medium. Cells were centrifuged at 1300 rpm for 5 min. The resulting cell pellet was resuspended in 2 ml of cell culture medium. Cells were counted using a haemocytometer and seeded into new flask at the concentration of 1 x 10^4. The process of detaching and seeding adherent cells from a culture to another one constitutes a "passage".

2.2.2 Cell cryopreservation and thawing

Cells were cryopreserved at -80 ºC in a cryopreservation medium consisting of a mixture of 90% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (DMSO).

For recovery of the cells, cryovials were rapidly thawed in the water bath at 37 ºC. Cells were immediately resuspended in 2 ml of fresh cell culture medium and centrifuged to remove DMSO. Cells were suspended in cell culture medium and seeded in flasks.

2.2.3 Haematoxylin and Eosin staining

After removing the culture medium, cells were washed with PBS 3 times. Brain cells were treated with 4% of paraformaldehyde solution to fix the samples. Then, cells were washed in PBS 3 times and. were permeabilised with 50 µL of 0.2% Triton X-100 solution. Then, cells were washed 3 times with PBS and with distilled water 2 times. Cells were stained with Haematoxylin for 3 minutes drop by drop directly on the cover glass of each well for 1 min at room temperature (RT). In order to wash the excess of Haematoxylin, cells were washed with tap water. Then, cells were treated with eosin for 45 seconds drop by drops. Immersion with 95% and 100% ethanol was used to remove the eosin dye. The slides were mounted by Eukitt.

2.2.4 Transmission electron microscopy (TEM)

Immortalized brain cells were seeded in 24-wells plates. At confluence, cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 ◦C. Samples were post-fixed with a mixture containing 1% osmium tetroxide and 1% potassium ferrocyanide in a 0.1 M sodium cacodylate buffer for 1 h at 4°C. After three washes with water, samples were

dehydrated by immersion in increasing concentrations of ethanol and embedded in epoxy resin (Sigma-Aldrich). Ultrathin sections (60–70 nm) were obtained with an Ultrotome V (LKB) ultramicrotome, counterstained with uranyl acetate and lead citrate. Samples were observed with a Tecnai G 2 (FEI) TEM operating at 100 kV and images were acquired with a Veleta digital camera (Olympus Soft Imaging System).

2.2.5 Immunocytochemistry

Immunocytochemical analyses were performed to characterize brain-derived cell line.
Cells are seeded in 24 well-plates containing sterile coverslips at a density of $1x10^4$ cells/well. When cells reached the 80% of confluence, were fixed with 4% of paraformaldehyde for 30 min at room temperature. After that, cells were washed with PBS to remove the paraffine residues. Cells were permeabilized with 50 µL of 0,1% Triton X-100 in PBS and washed with PBS twice. The cells permeabilization was followed by BSA 5% in PBS treatment for 30 minutes to block the non-specific endogenous sites. After that, cells were incubated overnight with the following primary antibodies at the specific dilutions:

- Ki-67 mouse 1:200 (Dako)
- Cytokeratin mouse 1:100 (GeneTex)
- Vimentin rabbit 1:200 (GeneTex)

The day after, cells were incubated for 2 hours with the following secondary antibodies at a concentration of 1:500:

- Alexa Fluor 488 goat anti-mouse (Biotium)
- Alexa Fluor Plus 647 goat anti-rabbit (Invitrogen)

Nuclei were stained with Hoechst 33342 (Sigma-Aldrich) with dilution 1:10.000 for 30 minutes. Coverslips were mounted on slides using Fluoromount. At least, the labelled cultures were observed under a Leica TCS SP5 confocal microscope.

2.2.6 Application of cold plasma treatment to a Bottlenose dolphin skin-derived cell line

Bottlenose dolphin skin-derived cell line was seeded in 96-well tissue culture plates at a concentration of 1 x 10^4 cells/well. After 24 hours, cells were exposed to cold atmospheric plasma treatment (CPT) for different Exposure time: 1, 2, 5, 6, 7, 8, 9 and 10 minutes, as showed in **Fig 1**. Non-treated cells were used as control. Cells were incubated at 37 ºC and 5% CO_2 for diverse Incubation period: 0 hours (T0), and after 8 hours (T8). Then, cells were fixed with 4% paraformaldehyde (PFA) following the time-point for the incubation period.

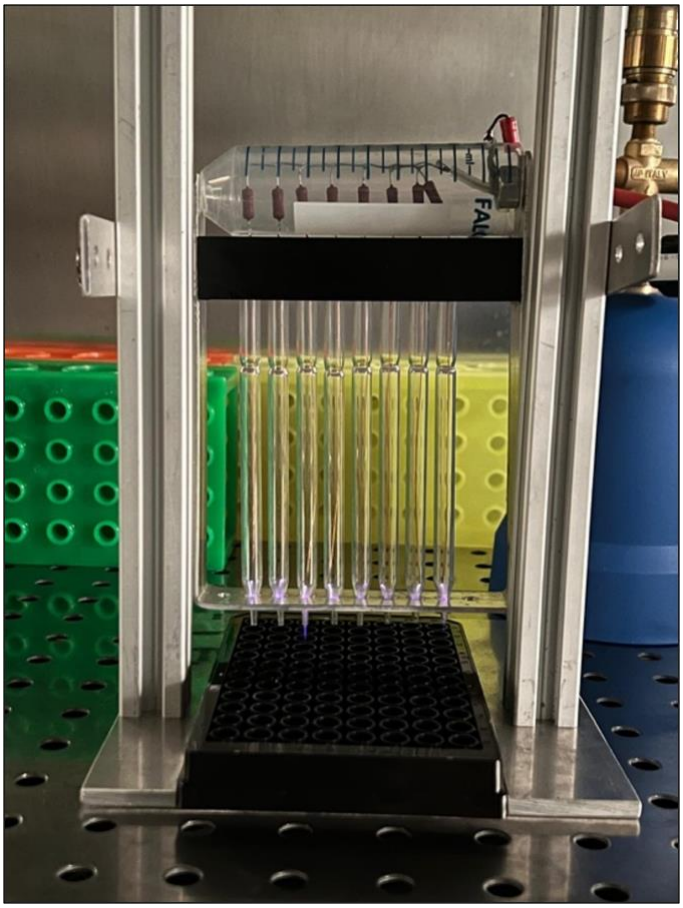


Fig 1: CAP treatment exposure on 96-well plates of skin-derived cells. The plasma purple jet is placed in a vertical position above the wells and the gas flow is 4 L/min.

2.2.7 Hoechst intensity characterization of Bottlenose dolphin skin-derived cell line

Cells were fixed with 4% of paraformaldehyde for 30 minutes. After incubation with paraformaldehyde, cells were washed twice with PBS 1x for removing residuals of paraformaldehyde. Cells were stained with Hoechst 33342 (ThermoFisher Scientific) previously prepared by diluting the Hoechst stock solution in PBS 1X. Then, cells were incubated for 30 min. Hoechst blue fluorescence was observed under confocal microscope.

2.2.8 MTT cell viability assay

Bottlenose dolphin skin-derived cell line was exposed to cold plasma treatment and the viability was evaluated using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The assay is based on the conversion of MTT into formazan crystals by living cells, which shows mitochondrial function.

Briefly, cells were seeded at a density of 1x 10^4 cells/well in 96-well microplates (Tissue Culture Plate 96-well, Sarstedt) and the cells were allowed adhered for 24 hours and incubated

at 5% CO2 at 37°C.Cells were exposed to cold atmospheric plasma treatment following the exposure time. Tapsigargin exposure was used as positive control at different concentration: 4 µM, 0,4 µM, 0,04 µM, 0,004 µM and 0,0004 µM. After treatment, cells were incubated with 5 mg/mL MTT dye solution for 2 h at 37° C. The incubation was stopped by adding 20 µL of stop solution in each well, containing sodium dodecyl sulphate (SDS) in order to solubilize the coloured formazan product. Plates were placed on a shaker overnight to assure complete solubilization of the formazan crystals Finally, the absorbance was measured at 590 nm with the multilabel plate reader VICTOR^{TM} X4 (PerkinElmer). As a readout for cell viability (%), the ratio between the absorbance of treated cells/absorbance of control cells * 100 was calculated.

2.2.9 Statistical methodology

In the statistical analysis was compared the control condition to each experimental condition tested. The distribution of the nuclei based on the nuclear morphological parameters was considered. After classification of cells in different groups according to their nuclear features, a series of nonparametric rotation tests (Solari et al., 2014) was performed, allowing comparison of each experimental condition versus control. The analysis performed was equivalent to a nonparametric one-to-many repeated measures ANOVA, where the repetitions are among plate and group. The statistical significance was defined by mean of a nonparametric Fisher test (Pesarin, 2001). The analysis has been performed with R software (R Core Team, 2021) and flip package (Finos, 2018).

3. RESULTS

3.1 CELL CULTURES ESTABLISHMENT AND CHARACTERISTICS

3.1.1 Bottlenose dolphin brain cell line characterization by haematoxylin and eosin staining and immunocytochemical analysis

Brain-derived cells stained with haematoxylin and eosin were analysed under optical microscope (**Fig. 2**). All cells presented centrally located, oval-shaped nuclei-stained dark pink, with purple heterochromatin granules inside them. Around the nucleus the cytoplasm stained

in a lighter pink, were in contact with other cells. Cells displayed irregular fusiform, bipolar, or multipolar morphologies and adhesive growth, typical of endothelial-like cells.

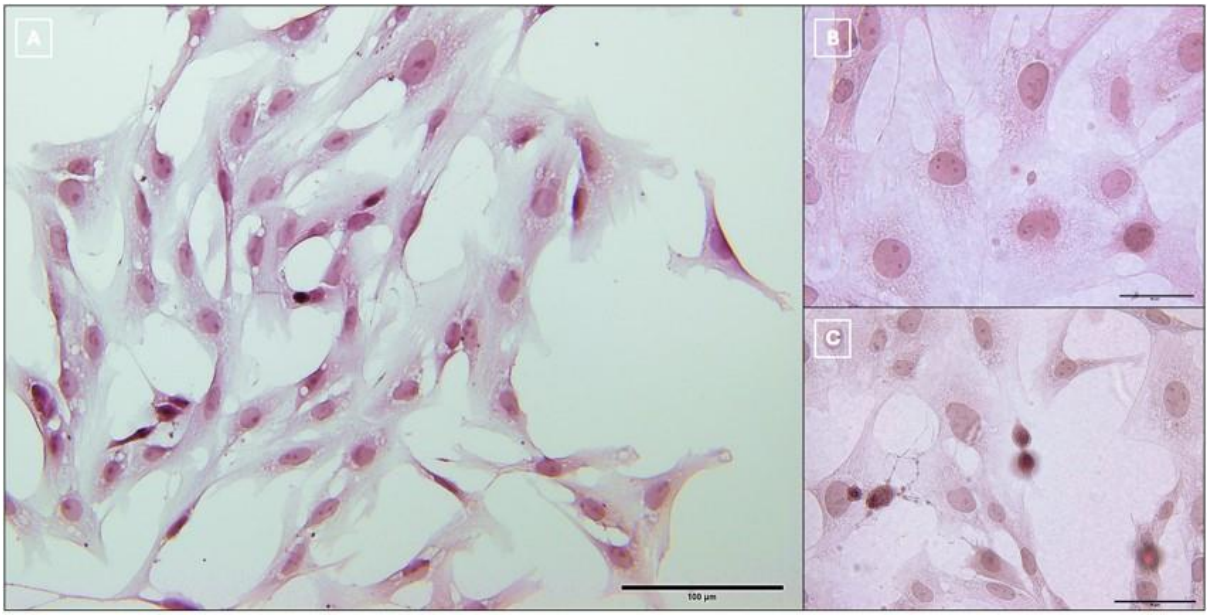


Fig. 2: Morphology of *Tursiops truncatus* brain-derived cells stained with haematoxylin and eosin and obtained with optical microscope. (A) scale bar= 100 µm. (B) scale bar= 50 µm. (C) scale bar= 50 µm

The characterization of Tursiops truncatus brain-derived cell line by immunocytochemical analysis targeting vimentin and Ki-67 is shown in **Figure 3** and **4**. The cell line revealed immunoreactivity (-ir) for vimentin, a cytoskeletal protein typically expressed in mesenchymal cells. The vimentin-ir cells showed cytoplasmic filamentous structures, consistent with the presence of intermediate filaments typical of fibroblast cells (**Fig. 2**). The cell line revealed immunoreactivity for Ki-67, a nuclear protein in proliferating cells. **Figure 4** showed evidence of chromosomes inside the nucleus. The cytokeratin is an intermediate cytoskeleton filament produced by cells of epithelial origin and in this case, no immunostaining to cytokeratin was detected (not shown).

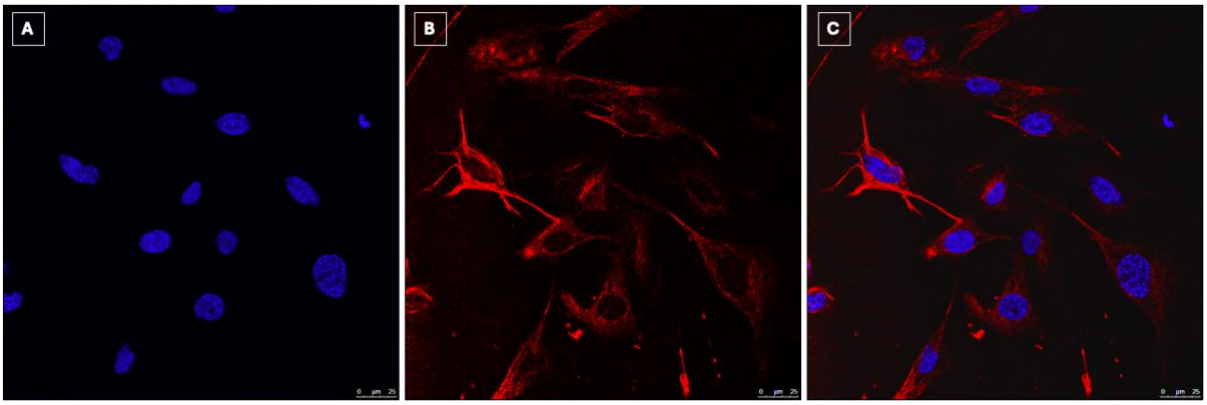


Fig. 3: Confocal images of brain cells. (A) Staining of cell nuclei with Hoechst (blue). (B) Staining of cell cytoplasmic filamentous structures with Vimentin-ir (red). (C) Merged of A and B images. Scale bar = 25 µm.

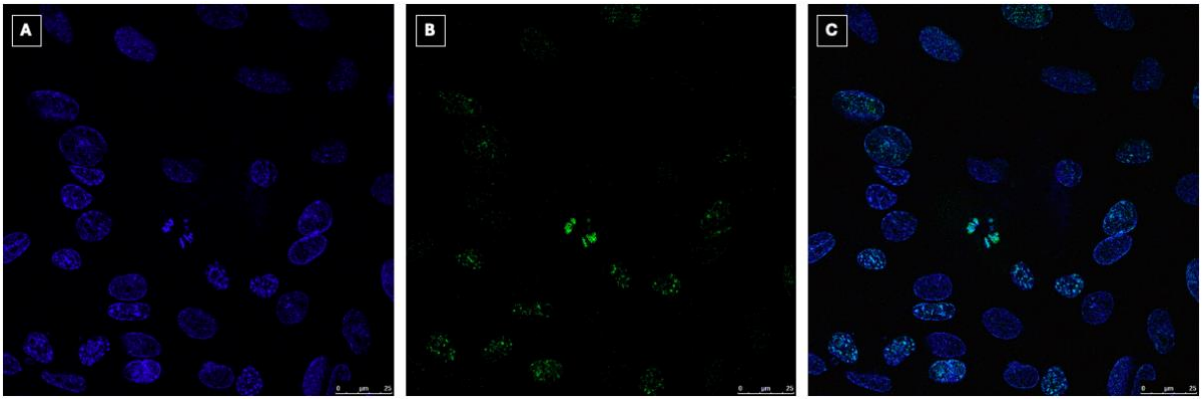


Fig. 4: Confocal images collected of brain cells display a nucleus during late mitosis, as the chromosomes are being forced apart by the mitotic spindle, indicating the end of mitosis and the start of cytokinesis. (A) Staining of cell nuclei with Hoechst (blue). (B) Staining of cells with Ki-67 (green). (C) Merged of A and B images. Scale bar = 25 µm.

3.1.2 Cells ultrastructure analysis by TEM

TEM analysis was used to reveal ultrastructural details of brain cell line. In general, the oval nuclei of all the examined cells showed a majority of euchromatin with a minor quantity of heterochromatin, indicating active transcription (**Fig 5A**). In the cytoplasm alongside the nucleus numerous mitochondria were detected, indicating of significant synthesis of proteins (**Fig. 5B**).

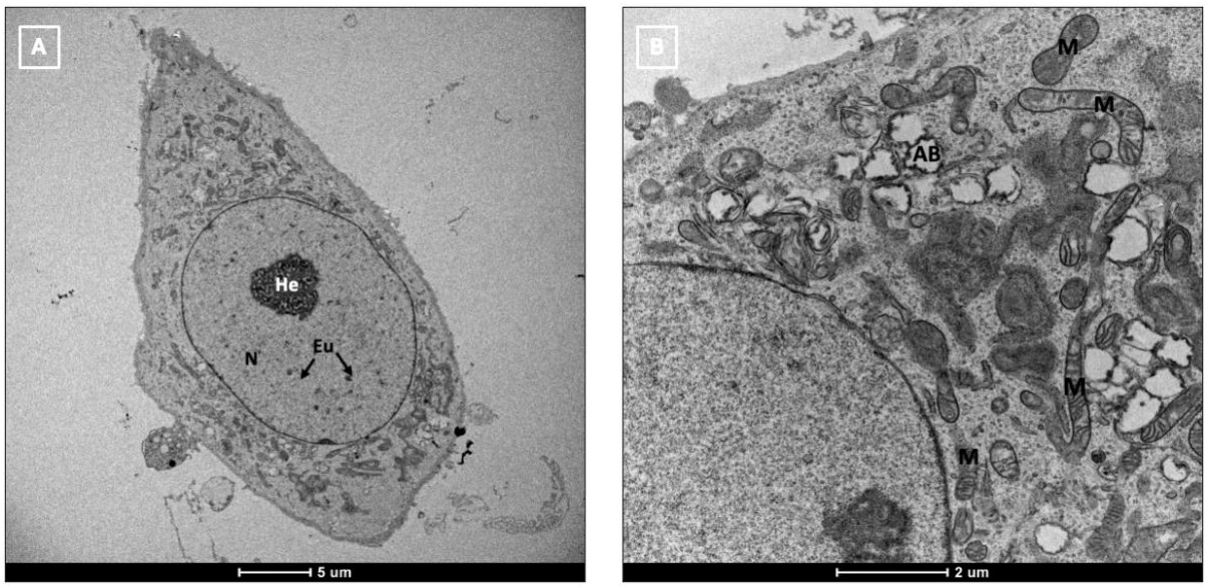


Fig 5: Details of TEM images of the brain-derived cells from *Tursiops truncatus*. **(A**) Oval nucleus is showed. Scale bar = 5 µm. (**B**) Enlarged micrograph of the same cell showing the presence of numerous mitochondria and autophagic bodies in the perinuclear region. Scale bar = 2 µm. Nuclei (N), containing heterochromatin (He) and mainly euchromatin (Eu). Mitochondria (M) and autophagic bodies (AB).

3.2 EFFECTS OF CAP ON BOTTELNOSE DOLPHIN SKIN-DERIVED CELLS

3.2.1 Preliminary results on Bottlenose dolphin skin-derived nuclei population after CAP treatment

Through a high-throughput screening analysis of the Hoechst-stained nuclei, we determine a preliminary study of how CAP inhibit the cell proliferation or increase the cell death in Bottlenose dolphin skin-derived cells compared to the control condition.

The data obtained from the HCS image analysis on skin-derived cells showed that CAP alter biological activity of the cells ($p<0.05$). The morphological parameters: nuclei length, nuclei intensity and nuclei ratio were combined to cluster cell nuclei into three population: the Normal population divided into 4 groups (G0, S, early M, late M), the Large population (apoptotic group) and the Small population (small nuclei fragments). The cell classification with the respective biological meaning and morphological parameters were reported in **Table 1**.

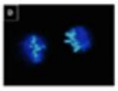
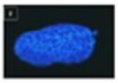
GROUP	NUCLEI POPULATION	NUCLEAR SHAPE	BIOLOGICAL MEANING	NUCLEAR LENGTH RANGE	NUCLEAR INTENSITY RANGE	NUCLEAR REGULARITY RANGE	EXAMPLE IMAGE
1. NORMAL REGULAR NUCLEI	NORMAL	Regular shape and size, weakly stained	Healthy cells (G0)	13-28 µm	1400-8500	0.5-1	
2. NORMAL REGULAR NUCLEI	NORMAL	Regular shape, intensely stained	DNA synthesis (S)	13-28 µm	8000-14000	0.5-1	
3. NORMAL IRREGULAR NUCLEI	NORMAL	Irregular shape, strongly stained	Early Mitotic phases	13-28 µm	14000-22000	0.2-0.7	
4. NORMAL IRREGULAR NUCLEI	NORMAL	Irregular shape, intensely stained	Mitotic phases	8-13 µm	4000-22000	0.2-0.9	
5. LARGE NUCLEI	LARGE	Regular shape, weakly stained	Cellular senescence, chromatin fading, mitotic catastrophe or nuclear damages	> 28 µm	1000-35000	0.2-0.5	
6. SMALL NUCLEI	SMALL	Irregular shape	Nuclear fragmentation condensed chromatin	<8 µm	2000-35000	0.2-0.9	
7. NEW GROUP		Irregular shape, intensely stained	Condensed chromatin	8-18 µm	22000-35000	0.2-0.9	

Table 1: Classification of the cell nuclei based on morphometric parameters. The three populations were divided into seven groups, each characterized by different nuclear/DNA morphology.

For each group the mean proportion of the cell nuclei was calculated, as showing **Figure 6**. In each group it is shown the major proportion of G0 cells of ~60%. It is evident that the number of nuclei belonging to the Small group and New apoptotic group is increasing with a CAP exposure time of 10 minutes and after 8 hours of incubation time, respectively by ~3% and ~10%. Noteworthy, when cells were exposed to 10 minutes of incubation time the S group decreased the number of cell nuclei with a proportion of ~7%.

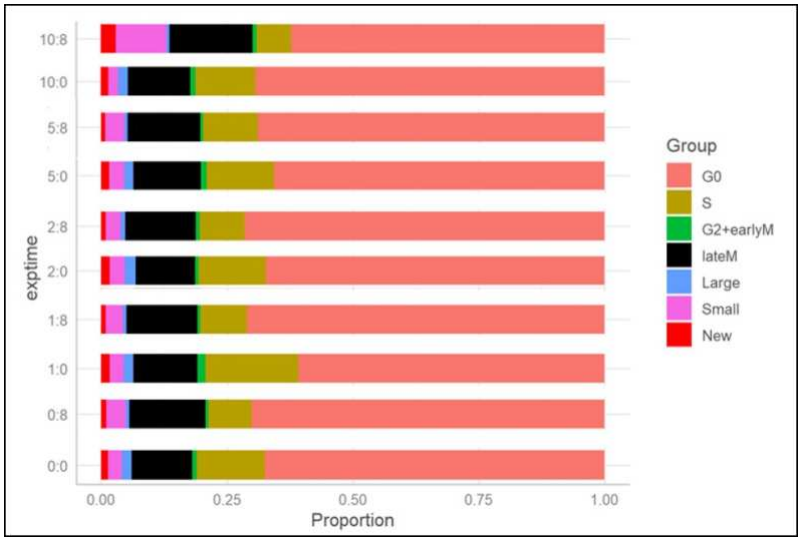


Fig 6: Bar charts showing the descriptive analysis of the mean proportion of nuclei for each group (G0, S, early M, late M, Large, Small fragments, Large and new apoptotic group) on the X-axis, at different exposure time of CAP (0 hour and 8 hour) compared to the control, on the Y-axis.

Interestingly, the inferential analysis revealed that at short-time exposure (1 min, 2 min, and 5 minutes), after an incubation time T0, CAP induced a statistically significant increase of cells in S phase at 1 minutes of exposure ($p<0.001$), in early M phase at 1 minutes of exposure ($p<0.003$) and at 2 minutes of exposure ($p<0.01$). Additionally, CAP exposure induced an increasing of cells belonging to Small group at 2 minutes of exposure ($p<0.003$) and at 5 minutes of exposure ($p<0.006$). It is statistically significant the enhance of cell nuclei of new apoptotic group at exposure time of 1 minutes, 2 minutes and 5 minutes.

Conversely, as detailed, in **Figure 7,** the CAP exposure at long-time exposure (6 min, 7 min, 8 min, 9 min and 10 minutes), and after incubation time T8, altered the cell cycle progression. In particular, CAP induced a decreasing of G0 cells at 6 min of exposure ($p<0.006$), at 7 minutes ($p<0.01$), at 9 minutes ($p<0.009$) and 10 minutes ($p<0.001$). Interestingly, the cells in S group, early-M group and late-M group decreased at all long-time exposure ($p<0.001$). No statistically significant evidence was showed in new apoptotic group at every exposure time.

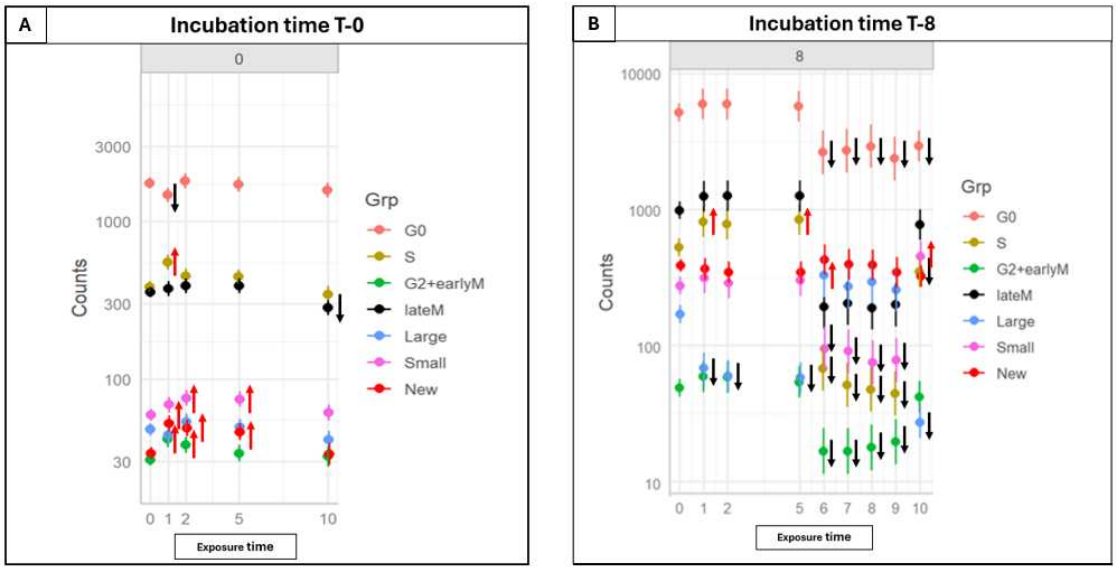


Fig 7: Boxplot showing the correlation between the exposure time (X-axis) and nuclei Counts (Y-axis) in each group of cell nuclei. (A) The boxplot shows the incubation time at 0 hours (T0) and the exposure time of 1 min, 2 min, 5 min and 10 min, compared to the control (0). (B) The boxplot shows the incubation time after 8 hours (T8) and the exposure time of 1 min, 2 min, 5 min, 6 min, 7 min, 8 min, 9 min and 10 minutes, compared to the control (0). The red arrows indicate a statistically significant increase ($p<0.05$) of cell nuclei per each group. The black arrows indicate a statistically significant decrease ($p<0.05$) of cell nuclei per each group.

3.2.2 MTT cell viability results on Bottlenose dolphin skin cell line

An MTT assay was performed in order to evaluate the impact of CAP exposure on cell viability. The Bottlenose dolphin's skin cell line incubation with Thapsigargin at a concentration of 4 µM showed a statistically significant decrease in cell viability compared to the control condition, in the incubation time analysed (T0 and T8). The decreasing in cell viability was evident in cells after the incubation time of 8 hours at a high exposure time to CAP (10 minutes), as shown in histogram B, in **Figure 8B**. No significant decrease in cell viability was determined at the lower concentration of Thapsigargin (0.4 µM, 0.04 µM, 0.004 µM and 0.0004 µM), as well as after CAP treatment at the incubation time T0 at different exposure time (1 min, 2 min, 3 min, 4 min, 5 min, 6 min, 7 min, 8 min, 9 min and 10 min) (**Figure 8A**).

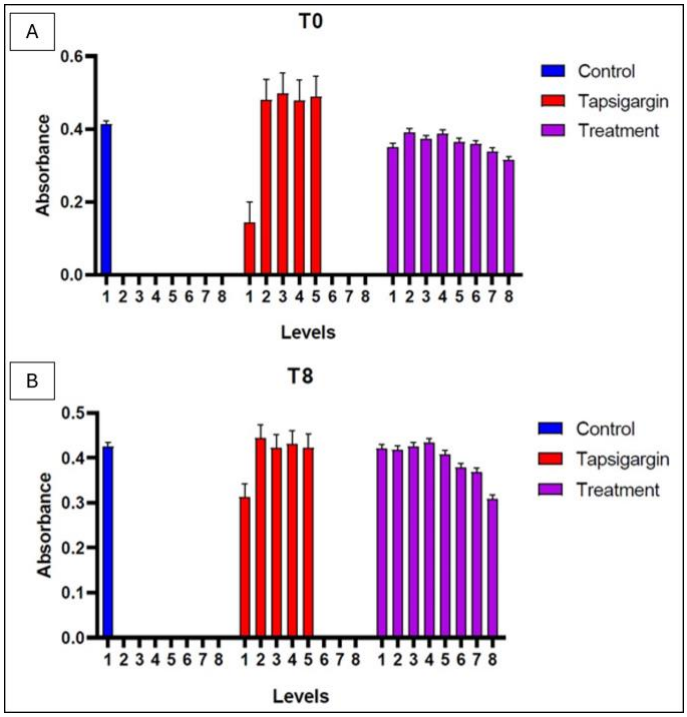


Figure 8: Cell viability evaluation by means of MTT assay of skin cells exposed to CAP treatment at diverse exposure time: 1=1 min, 2=2 min, 3=5 min, 4=6 min, 5=7 min, 6=8 min, 7=9 min and 8=10 min.(A) Bar charts showing the incubation time at 0 hours. (B) Bar charts showing incubation time at 8 hours. Thapsigargin was used as positive control at different concentrations: 1=4 µM, 2=0,4 µM, 3=0,04 µM, 4= 0,004 µM and 5=0,0004 µM

4. DISCUSSION AND CONCLUSIONS

4.1 Characterization of brain-derived cetacean cell line

To overcome ethical and legal constraints, conduct long-term studies on the impact of pollutants, and enable in vivo investigations on protected cetacean species, the establishment and characterization of stable cetacean cell lines are essential. These cell lines provide a practical alternative since in vivo investigations on protected cetacean species are almost impossible to perform.

The characterisation and maintenance of the brain -derived cell line obtained from a cetacean belonging to the family Delphinidae (Bottlenose dolphins) are successfully reported in this thesis. From a morphological point of view based on their appearance, haematoxylin and eosin staining, TEM ultrastructural examination, and immunohistochemistry investigation, the immortalized cells were largely identified endothelial cells.

We choose three protein markers to determine and identify the nature and the type of our brain-derived cells. The immunocytochemical analysis revealed that all cells are vimentin-ir. The vimentin is an intermediate filament protein, typically expressed by mesenchymal cells, including endothelial cells and fibroblasts. As expected from the presence of intermediate

filaments characteristics of fibroblast cells, the vimentin-ir cells displayed cytoplasmic filamentous structures (Cheng et al., 2016).

The second marker used is the protein Ki67. It has a short half-life and is capable of binding to the DNA of actively replicating cells, primarily located in the nuclear region during the S phase and mitosis phase, where its expression is at its highest, while it is not detectable during the G0 phase (Sun and Kaufman, 2018). The presence of Ki67 has been confirmed to be essential for the efficient completion of the cell cycle and, consequently, for proper proliferative activity of the cell. Therefore, it represents another important marker which confirm the health status of our cell line.

4.2 Effects of CAP applied to skin-derived cetacean cell line: a preliminary study

The preliminary results of this thesis investigating the effects of cold atmospheric plasma (CAP) treatment on a skin-derived cell line of Bottlenose dolphin (*Tursiops truncatus*) reveal significant insights into the cellular responses to varying exposure time of plasma source. Our results indicate a marked decrease in cell viability corresponding to long-exposure time (6 min, 7 min, 8 min, 9 min and 10 minutes), particularly in cells undergoing mitosis, S phase, and G0 phase. This trend is consistent with prior studies that have examined the effects of CAP on various cell lines. For instance, Keidar et al. (2011) reported that CAP can induce apoptosis and reduce cell proliferation in human glioma cells, suggesting that plasma exposure disrupts the cell cycle and promotes cell death through oxidative stress mechanisms. Similarly, Cheng et al. (2016) demonstrated that CAP treatment led to a significant reduction in the viability of melanoma cells, correlating with increased DNA damage and cell cycle arrest.

In this thesis, the observed reduction of mitotic cells suggests that CAP treatment may prevent cell division, potentially through the induction of DNA damage or disruption of mitotic spindle formation. The decrease in S phase cells further supports the hypothesis that CAP interferes with DNA replication processes. Moreover, the reduction in G0 phase cells implies that quiescent cells are not spared from plasma-induced stress, potentially leading to apoptosis or senescence. These effects can be attributed to the generation of reactive oxygen species (ROS) produced by CAP, which are known to cause oxidative damage to cellular components, including DNA, proteins, and lipids. The sensitivity of cells to CAP appears to be dependent on the duration of exposure, with longer treatments resulting in more pronounced cellular damage and decreased viability. Our results align with the findings of Ahn et al. (2014), who observed that CAP treatment led to significant cytotoxic effects in keratinocytes and fibroblasts, with long-time exposure exacerbating the damage.

Conversely, cells exposed to short-time CAP exposure (1, 2, and 5 minutes) and incubation time of 0 hours, showed an increase in the number of cells in mitosis, S phase, and nuclear fragments. These findings suggest that short-term CAP exposure may stimulate cellular activity and proliferation, potentially through transient activation of cell cycle regulatory pathways. This is consistent with findings by Yan et al. (2017), who reported that short-time CAP exposure could enhance proliferation in cells by modulating oxidative stress levels and activating cell survival pathways. Moreover, an increase in S phase cells indicates that CAP might accelerate DNA replication processes, promoting cell cycle progression. Interestingly, the increasing of nuclear fragments could imply that while CAP promotes proliferation, it also induces a degree of sub-lethal stress, leading to partial nuclear disintegration. This aligns with the investigations of Kim et al. (2011), who observed that CAP could induce DNA damage and repair mechanisms, contributing to increased cellular turnover and proliferation. The balance between CAP-induced proliferative signals and DNA damage response might explain the dual observation of increased cell numbers and nuclear fragments. In conclusion, our preliminary investigation of the effects of CAP on a skin-derived cell line of Bottlenose dolphin reveals a time-dependent decrease in cell viability, particularly affecting cells in G0, S and mitotic phase. On the other hand, optimizing plasma short-time exposure parameters will be crucial for minimizing adverse effects and maximizing therapeutic efficacy. Further studies should focus on the investigation of the potential therapeutic applications of CAP and its application to new in vitro models, exploiting their versatility.

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