„ALEXANDRU IOAN CUZA” UNIVERSITY OF IAŞI
FACULTY OF BIOLOGY
DOCTORAL SCHOOL OF BIOLOGY

The evaluation of non-thermal atmospheric pressure plasma treatment effects on *in vitro* cellular models

PHD THESIS SUMMARY

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Special thanks

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List of abbreviations

AC – alternative current

ACTB – β-actin

ADN – deoxyribonucleic acid

ADNc – complementary deoxyribonucleic acid

APPJ – atmospheric pressure plasma jet

ARN – ribonucleic acid

ARNm – messenger ribonucleic acid

BAX – BCL2 Associated X, Apoptosis Regulator

BCL2 – BCL2 Apoptosis Regulator

CAP – cold atmospheric plasma

CASP3 – caspase 3

CASP8 – caspase 8

CASP9 – caspase 9

CFLAR – CASP8 And FADD Like Apoptosis Regulator

CO_2 – carbon dioxide

Ct – cycle threshold

DBD – dielectric barrier discharge

DCFH – dichlorofluorescin

DCFH-DA – dichlorodihydrofluorescein diacetate

DIABLO – Diablo IAP-Binding Mitochondrial Protein

DMSO – dimethyl sulfoxide

DPBS – Dulbecco phosphate buffered saline

Ds – double-stranded

DSB – double-strand breaks

EDTA – ethylenediaminetetraacetic acid

GE – grounded electrode

HNO_2 – nitrous acid

HNO_3 – nitric acid

HOB – human osteoblasts

HOS – human osteosarcoma

HVE – high-voltage electrode

JNK1 – C-Jun N-Terminal Kinase 1

MKI67 – Ki-67 proliferation marker

MTT – 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

NO – nitric oxide

NO_2^- – nitrite

NO_3^- – nitrates

OES – optical emission spectroscopy

OS – osteosarcoma

PAM – plasma activated medium

PAM-PBS – plasma activated phosphate buffered saline

PAM-RPMI – plasma activated Roswell Park Memorial Institute medium

PBS – phosphate buffered saline

PCR – polymerase chain reaction

RE – endoplasmic reticulum

RIPK3 – Receptor Interacting Serine/Threonine Kinase 3

RNS – reactive nitrogen species

RONS – reactive oxygen and nitrogen species

ROS – reactive oxygen species

RPMI 1640 – Roswell Park Memorial Institute 1640 medium

RT-qPCR – quantitative real time polymerase chain reaction

SDS – sodium lauryl sulfate

TAE – tris-acetate EDTA buffer

Texp – exposure time

TNFα – tumor necrosis factor alpha

TP53 – tumoral protein p53

TRIS – tris(hydroxymethyl)aminomethane

Ttr – treatment time

Tx – paclitaxel

UV – ultraviolet radiation

UVA – type A ultraviolet radiation

UVB – type B ultraviolet radiation

UVC – type C ultraviolet radiation

XIAP – X-Linked Inhibitor Of Apoptosis

THEORETICAL PART
Introduction

Cancer is one of the most widespread diseases nowadays, with a significant impact on the quality of life, healthcare budgets, and a profound emotional impact on both the patient and their family (Park & Look, 2019)The uncertainty regarding the overall effectiveness of current anti-tumor treatments, as well as cancer recurrence due to acquired resistance to used drugs, are two major issues of current therapeutic strategies in combating this disease. Therefore, there is a continuous trend of identifying new methods or strategies to combat cancer. (Gerber et al., 2017).

In the last decade, cold atmospheric plasma (CAP), a partially ionized gas with a temperature close to room temperature, has shown promising results in cancer therapy, demonstrating its beneficial applicative nature in the attempts to treat this disease (Yan et al., 2017). The main scientific studies on the effects of non-thermal atmospheric pressure plasma (CAP) on various types of neoplastic cells have shown that non-thermal atmospheric plasma is responsible for inducing apoptosis (Arndt et al., 2013; Chang et al., 2014; Ninomiya et al., 2013; Thiyagarajan et al., 2014), necrosis (Elaissi & Charrada, 2021; Yan et al., 2020), necroptosis (Yang et al., 2020), autophagy (Yan et al., 2021; Yoshikawa et al., 2020), ferroptosys (A. Jo et al., 2022; Nguyen et al., 2023), pyroptosis (Motaln et al., 2021) and for blockage of the cell cycle (Maes et al., 2017) by generating reactive oxygen species or reactive nitrogen species. An important characteristic of CAP is its selective activity on neoplastic cells with a reduced negative impact on normal cells (Keidar et al., 2011, 2013). Also, CAP causes detachment of tumor cells and reduces the migration speed of the cells (Kong et al., 2011), as well as inhibiting metastatic processes (Chang et al., 2014).

However, the molecular mechanisms behind the selectivity and induction of apoptosis through CAP treatment are not fully identified yet.

The motivation, purpose and objectives of the paper

Motivation. In 2019, cancer surpassed cardiovascular diseases in terms of the number of deaths per year, causing the death of approximately 10 million people (Lin et al., 2021). Statistical analyses and specialized studies predict that cancer will soon become the leading cause of death in every country around the globe (Lin et al., 2021). Annually, in Europe, the costs associated with cancer amount to an average of approximately 100 dollars per capita (Haier & Schaefers, 2022). Despite technological advancements and the pharmaceutical industry's progress, cancer continues to be a devastating disease with high mortality rates (Faramarzi et al., 2021).

In the mid-2000s, due to its demonstrated antitumor properties, cold atmospheric plasma caught the attention of researchers in the biomedical fields as a potential new method for cancer therapy (Dubuc et al., 2018). Although significant progress has been made in identifying the mechanisms of interaction between CAP and cells or tissues, further studies are still needed to identify the underlying mechanisms by which CAP treatments exert their antitumor properties (Murillo et al., 2023).

Aim. In the context presented earlier, the aim of this work is to study the effects of non-thermal atmospheric pressure plasma on experimental *in vitro* models (normal and tumor cell cultures) to assess its potential for selective cytotoxicity and identify the cellular mechanisms involved in plasma-cell interactions.

The objectives of this thesis.

O.1. Identification of the optimal exposure conditions of cell cultures to cold atmospheric plasma.

O.2. Temporal evaluation of the effects of cold atmospheric plasma treatment on normal and tumor cell cultures.

O.3. Evaluation of the selective nature of cold atmospheric plasma.

O.4. Identification of the cellular signaling pathways responsible for activating mechanisms of cell survival or cell death, modulated by cold atmospheric plasma treatment.

O.5. Dissemination of results - publishing in specialized journals and participating in scientific events.

O.6. Writing and defending the doctoral thesis.

1. Cold atmospheric plasma and its importance in biomedicine.

Plasma is a partially ionized gas composed of ions, electrons, photons and neutral elements, which are active species capable of inducing various physical phenomena and chemical reactions. Considered the fourth state of matter, plasma can be found in nature (plasma created in stars, aurora borealis, lightning, etc.), but it can also be generated under laboratory conditions by applying an external source of energy to a neutral gas, which becomes an electrical conductor. During plasma formation, energy is transferred to the gas in which the discharge occurs, creating large amounts of ions and electrons. These electrons will induce several reactions leading to molecular ionizations or dissociations. Consequently, the plasma produced will be composed of a mixture of reactive species generated as a result of these interactions (Fridman et al., 2008; Hoffmann et al., 2013; H. Jo et al., 2016; Kalghatgi et al., 2011).

Plasmas can be divided into two different types, depending on the thermal balance between ions and electrons, namely thermal and non-thermal plasmas (or cold). Thermal plasmas are those in which electrons have the same temperature as ions, and non-thermal plasmas, also known as cold plasmas, are characterized by the fact that electrons have a higher temperature than ions and neutrals, their temperature being close to room temperature (Stoffels et al., 2008; von Woedtke et al., 2013). The mass difference is so substantial that electrons can reach temperatures ranging from a few thousand to tens of thousands of degrees Celsius, while the plasma as a whole remains roughly at room temperature (Heinlin et al., 2011).

Non-thermal plasmas are mainly composed of reactive nitrogen and oxygen species, UV radiation and charged particles, their composition being dependent on the

type of feed gas used, applied voltage, device type, physical configuration or exposure time (Bekeschus et al., 2013; Weltmann & von Woedtke, 2017).

1.1. Types of cold atmospheric plasma and treatment

One of the most widespread methods of cold atmospheric plasma (CAP) generation is dielectric barrier discharge (DBD). (Laroussi & Akan, 2007). Regardless of the configuration, with one or two electrodes, two types of DBD are distinguished: **DBD without flow** in which the power electrode is wrapped with an insulating material and the plasma remains confined in the space between the electrodes, and **DBD with flow** in which the plasma is located both in the space between the electrodes and in the post-electrode region (Judée et al., 2019).

Regarding the treatment of tumor cells, two CAP treatment methods are widely used, namely direct treatment and indirect treatment. In direct treatment, cells are directly subjected to CAP discharges, while in indirect treatment, a liquid is subjected to discharges, which is then administered to the cells (Malyavko et al., 2020).

1.2. History of plasma usage in biomedical studies

1.2.1. Decontamination of surfaces

In the mid-1990s, experiments were conducted that showed that non-thermal atmospheric pressure plasma (CAP) could be used to inactivate bacteria (Laroussi, 2018). In the food field, CAP represents a promising method for the microbial decontamination of food, work surfaces, packaging, and processing equipment (Min et al., 2016; Patange et al., 2018).

1.2.2. Blood coagulation

There are multiple theories regarding the biochemical mechanisms of CAP-induced blood coagulation, with various studies demonstrating the direct effect of CAP

on blood proteins, this treatment causing coagulation without altering Ca^{2+} concentration or pH (Heslin et al., 2014).

1.2.3. Effects on wound healing

Cold atmospheric plasma treatment can have the effect of lowering the pH, leading to wound acidification, this therapeutically induced process supporting the healing process (Martinez et al., 2019; Mirpour et al., 2020).

1.2.4. Treatment of atopic eczema

In order to treat atopic dermatitis with CAP, Mertens et al. identified an antiseptic effect, reduction of skin redness, as well as a significant reduction in itching for several hours, with *Staphylococcus aureus* colonies reduced more than ten-fold (Mertens et al., 2009).

1.2.5. Skin regeneration technology using plasma

In 2005, atmospheric pressure non-thermal plasma skin resurfacing technology was approved for the treatment of facial wrinkles, superficial skin lesions, actinic keratoses, seborrheic keratoses and viral papilloma (Bogle et al., 2007).

1.2.6. The role of CAP in antitumor therapy

Since the first report of the induction of cell death by DBD in melanoma in 2007, the field of application of CAP in cancer treatment has seen rapid growth. To date, CAP treatment has demonstrated its anti-tumor ability on numerous cancer cell lines, the most intensively investigated cancers being brain cancer, skin cancer, breast cancer, colorectal cancer, lung cancer, cervical cancer, leukemia, hepatoma, and head and neck cancer (Dubuc et al., 2018).

1.3. Changes induced by CAP treatment at the cellular and molecular level

The main therapeutic effects of CAP are associated with the presence of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by CAP systems, these interacting with the cell membrane, causing morphological changes (Yan et al., 2017). At the molecular level, DNA damage has been observed in the early stages of CAP treatment (Gay-Mimbrera et al., 2016). CAP treatments can also result in lipid peroxidation and increased cytoplasmic calcium concentration (Görlach et al., 2015). Another consequence observed as a result of CAP treatment is the reduction of cell adhesion to culture vessels, migration and invasion (Lee et al., 2009).

1.4. The use of cold atmospheric plasma in the osteosarcoma therapy

Similar to the observations on other types of cancer treated with CAP, also in the case of OS the treatment had the effect of inducing oxidative stress (Ermakov et al., 2021; Mateu-Sanz et al., 2020; Xu et al., 2020). Using both different types of CAP generation devices and different methods and different treatment times, studies have demonstrated the ability of CAP to reduce the viability or proliferative potential of several types of OS cell lines (Canal et al., 2017; Gümbel, Suchy, et al., 2017; Nitsch et al., 2023; Stache et al., 2023; Xu et al., 2020). Some studies have identified OS cells treated with CAP as being in various stages of apoptotic or cell cycle arrest by evaluating p53 protein, caspases 3 and 7 levels, as well as cell morphology and nuclear fragmentation (Canal et al., 2017; Gümbel, Bekeschus, et al., 2017; Gümbel et al., 2016).

2. Markers of cell cycle control and cell death mechanisms

In order to identify the signaling pathways modulated by the indirect treatment with CAP, in this study the expression level of: six pro-apoptotic genes (BAX, JNK1, DIABLO, CASP8, CASP9 and CASP3), three anti-apoptotic genes (BCL2, CFLAR and

XIAP), two necroptotic genes (TNFα and RIPK3), two genes involved in cell cycle control (TP53 and CDKN1A) and one gene involved in cell proliferation (MKI67).

EXPERIMENTAL PART
3. Materials and working methods

3.1. Experimental design and PAM generation

The physical devices and measurements were carried out in the IPARC laboratory (Iasi Plasma Advanced Research Center), Faculty of Physics, "Alexandru Ioan Cuza" University from Iași, under the guidance of Mr. conf. univ. dr. habil. Ionuț Topală and Mr. CS III dr. Ilarion Mihăilă.

The experimental plan of the present study consisted of three main work stages: 1. Evaluation of the pH of PAM solutions and establishment of the effectiveness of PAM-PBS and PAM-RPMI solutions in terms of cytotoxicity; 2. Analysis of the physical discharge parameters and the content of RNS in the PAM; 3. Optimization of unloading parameters and evaluation of PAM-RPMI effects on tumor and normal cells, establishment of pH influence, analysis of gene expression of interest and assessment of intracellular RONS.

The CAP generation DBD system consists of a high-voltage alternating current (AC) power supply, produced in the IPARC laboratory, with a sinusoidal waveform (maximum voltage 15 kV), a high-voltage electrode (HVE), a dielectric layer and a ground electrode (GE).

In vitro experiments were performed by indirect exposure of tumor and normal cells, which involves treating them with activated liquid medium (PAM) beforehand with non-thermal plasma at atmospheric pressure type DBD without flow. In this study, the solutions exposed to the plasma were the culture medium RPMI 1640 and phosphate buffer saline, the exposure times of these two solutions to CAP being 30, 60, 90 or 120 seconds, and the treatment times of the cells with PAM were between 5 and 30 minutes, depending on the experiment.

3.2. Electrical characterization of CAP discharges

The voltage and current characteristics of CAP discharges were analyzed using Tektronix P6015A and Pearson 6585 probes connected to TDS5034 digital oscilloscope. The optical emission spectra of the CAP constituents were detected using a monochromator equipped with a CCD detector (Horiba Triax 550 with Symphony CCD detector) in a wavelength range between 300 nm and 430 nm. The temperature variation of the PAM solutions and adjacent wells during the exposures was measured using a multimeter and a K-type thermocouple.

3.3. pH analysis

The pH determinations of the PAM solutions were made using the PH 210 Microprocessor pH Meter, by evaluating 3 ml of solution from each experimental variant.

3.4. In vitro cell model and CAP treatment

In order to evaluate the biological effects of CAP treatments, two standardized cell lines were used in this study, namely a human osteosarcoma cell line (HOS) and a human osteoblast cell line (HOB). Indirect CAP treatments were performed in 96-well cell culture plates by removing the growth medium, washing the cells with PBS, applying 100 µl of PAM/well, removing it after a period of time (known as treatment time) ranging from 5 to 30 min (depending on the experiment), and adding 100 µl of growth medium/well. The in vitro cell experiments were carried out in the cell culture department, within the Center for Fundamental Research and Experimental Development in Translational Medicine (TRANSCEND) of the Regional Oncology Institute from Iași.

3.5. Isolation and purification of RNA

RNA isolation was achieved using the Tri-Reagent reagent (cat. 93289-100ML, Sigma Aldrich, MO, USA), using the phase separation by centrifugation method.

3.6. Standards preparation

For the quantification of gene expression, a set of standards, of known concentrations, serially diluted, is used to generate a standard curve. These were carried out by the reverse transcription of previously extracted RNA samples, the PCR amplification of the fragments of interest (using specific primer sets), the electrophoretic validation of the amplicons in agarose gel, their purification and quantification and the realization of serial dilutions, for each gene to be analyzed separately.

3.7. Quantification of gene expression involved in cell cycle control and cell death mechanisms by RT-qPCR

Gene expression quantification was performed by the RT-qPCR method, using the commercial kit GoTaq® 1-Step RT-qPCR System (Promega, Wi, USA) and the Rotor Gene 6000 Q 5-plex HRM amplification and detection system (Corbett Research, QIAGEN Corporation, GERMANY). Ct values for each individual assay were determined automatically by the data acquisition program based on amplification curves of standard samples. Later, the data were normalized using the Ct values of the reference gene ACTB and related to the values of the samples from the control groups, by the $2^{-\Delta\Delta Ct}$ method. Finally, the results were expressed in the form of $\log_2 2^{-\Delta\Delta Ct}$.

3.8. Cell viability assay

In order to establish the cytotoxicity of the treatment, MTT colorimetric viability tests were carried out, which are based on the metabolism by living cells of the

tetrazolium salt producing formazam, an insoluble blue compound which, by solubilization with DMSO, can be detected spectrophotometrically at a wavelength of 570 nm.

3.9. Evaluation of reactive oxygen and nitrogen species

3.9.1. Analysis of reactive oxygen species

In order to determine the levels of intracellular ROS, the fluorescent detection method with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (cat. D6883-50MG, Sigma Aldrich, MO, USA) was used. It enters the cell interior where it is rapidly hydrolyzed by cellular esterases to non-fluorescent DCFH, which is retained in the cell. Oxidation of DCFH by hydrogen peroxide or other reactive oxygen species produces the fluorescent indicator dichlorofluorescein (DCF) which was detected using the FilterMax F5 multimodal plate reader (Molecular Devices, CA, USA).

3.9.2. Analysis of reactive nitrogen species

Intracellular and PAM RNS levels were determined using the Griess assay (Griess Reagent System, cat. G2930, Promega, WI, USA). The principle of the method is based on the reaction under acidic conditions of nitrite with sulfanilic acid, forming the diazonium salt. This reacts with the aromatic amine 1-naphthylamine to produce a red-violet azo dye that was detected using the FilterMax F5 multimodal plate reader (Molecular Devices, CA, USA).

4. Results and discussion

4.1. Electrical characterization of DBD without flow discharges

Figure 4.1 shows a typical current-voltage waveform obtained during operation of the CAP generating source. From the electrical point of view, the discharges from the dielectric barrier are presented in the form of packets with the frequency of 100 Hz, the frequency of the electrical voltage identified in the packets was 23 kHz.

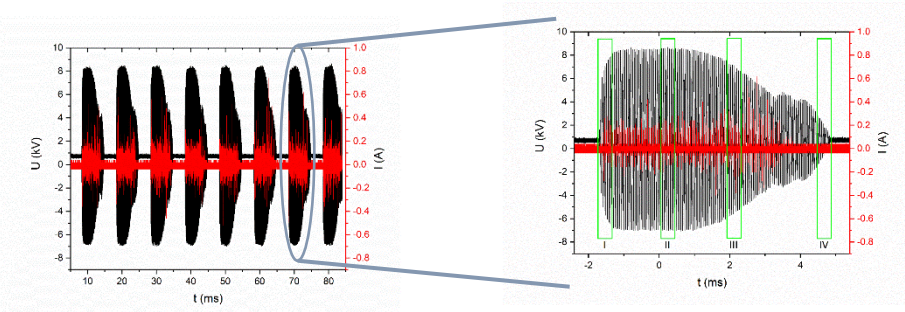

Figure 4.1. Dielectric Barrier Discharge (DBD) pulse pattern and current-voltage waveforms in a discharge package

4.2. Optical emission spectroscopy

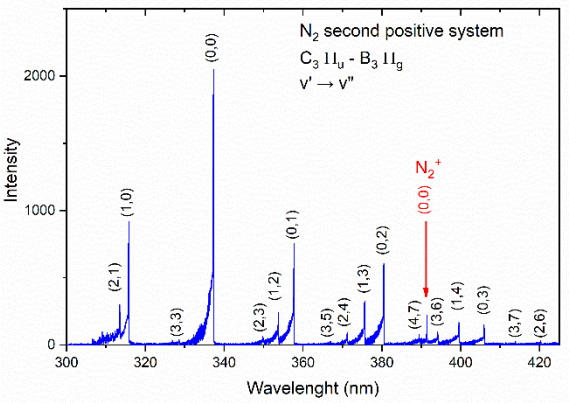

Figure 4.2. The optical emission spectrum of cold atmospheric plasma (CAP) in the range 300 – 427 nm

The optical emission spectra obtained for the CAP discharges show several characteristic lines of the second positive system of molecular nitrogen (Figure 4.2), identified at wavelengths 313.51, 315.78, 328.55, 337.28, 349.86, 353.73, 357.67, 366.89, 371.21, 3 75.72, 380.48, 389.43, 391.46, 394.15, 399.56, 405.92, 413.91 and 420.22 nm.

4.3. Exposure temperature

4.3.1. Discharge temperature

To determine the rotation temperature of nitrogen during the CAP discharge, high-resolution spectra were obtained in the range 331 - 338 nm (with a resolution of 0.017 nm). The obtained OES data were analyzed using the second positive C-B system of molecular nitrogen as a simulation model for data fitting with MassiveOES software (Voráč et al., 2019 identifying an average rotation temperature value of 1163 ± 24 K.

4.3.2. PAM temperature

By monitoring the temperature of the solution subjected to the CAP discharges, it was observed that the exposure caused only minor temperature increases of the PAM, which did not exceed a value of 30°C.

4.4. Evaluating the pH level of PAM

Observing the gradual color change of RPMI 1640 medium (containing phenol red) during exposures to CAP discharges, depending on the exposure time used, the next step of the study was to evaluate the pH of the obtained PAM solutions. Following the exposure of PBS and RPMI solutions to CAP for 30, 60, 90 and 120 seconds, a gradual acidification of these was identified in a dose-dependent manner (Figure 4.3). According to the obtained results, the acidification process was more pronounced in the case of PAM-PBS solutions.

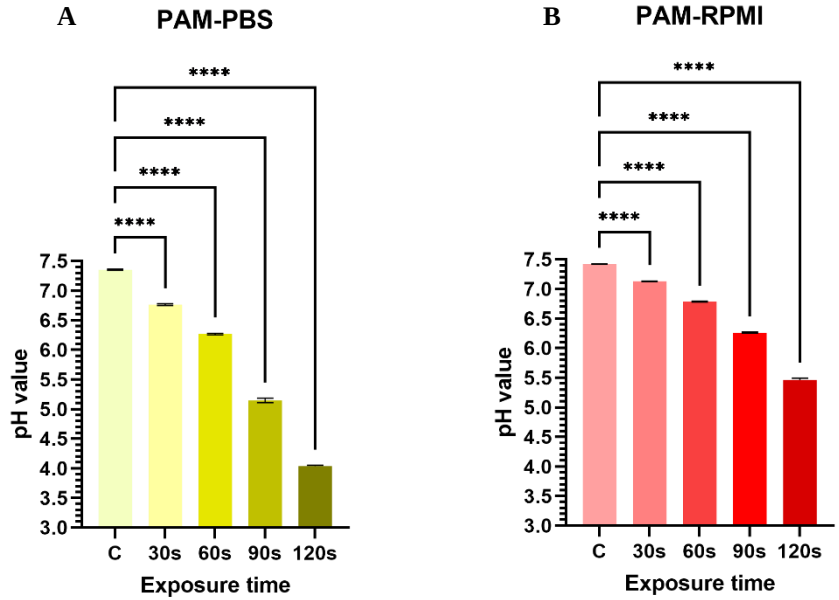

Figure 4.3. pH analysis of PAM-PBS (A) and PAM-RPMI (B) solutions after exposure to CAP. Values are mean±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (**** $p<0.0001$).

4.4. PAM pH stability at different storage temperatures

PAM-PBS treatments show fluctuations in pH levels between 0.01 and 0.33, which are not influenced by storage temperature. In the case of PAM-RPMI treatments, the fluctuations of the identified pH levels were between 0.03 and 0.46, unlike PAM-PBS, these fluctuations being dependent on the storage temperature. The obtained results demonstrate that both types of treatments can be stored for up to 3 months, the pH changes recorded not being large enough to influence the results of indirect treatments with CAP (Figure 4.4). However, it can be observed that −80°C is the optimal storage temperature of the PAM-RPMI treatment due to its variation below 0.1 pH, for all experimental variants.

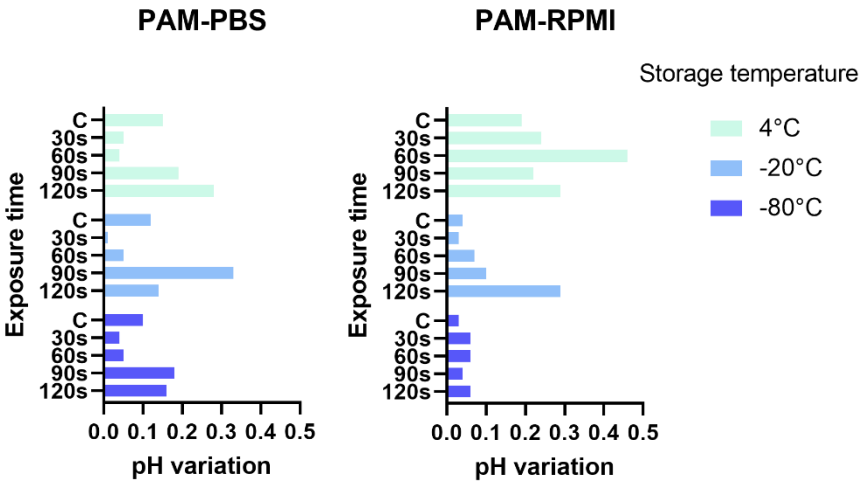

Figure 4.4. Variations of pH values of PAM-PBS and PAM-RPMI solutions identified during 3 months of storage at different temperatures.

4.5. Impact of PAM-PBS and PAM-RPMI treatments on cell viability

The results obtained indicated that, for exposure times of 30 seconds and 60 seconds, the cytotoxic effects were relatively comparable between the two solutions, with a slightly stronger cytotoxic effect observed for PAM-RPMI. However, for exposure times of 90 seconds and 120 seconds, a significant difference was observed between the two solutions, with PAM-RPMI 90s resulting in a 78% and PAM-RPMI 120s 85% reduction in HOS cell viability, while PAM-PBS 90s and PAM-PBS 120s treatments reduced cell viability by only 17.2% and 26.5%, respectively (Figure 4.5). The possible reactivity between CAP and the multitude of constituents of the RPMI medium, as opposed to the plain PBS solution, may be the cause of the higher cytotoxicity observed in this study when using PAM-RPMI. As a result of the higher degree of cytotoxicity, RPMI 1640 was selected as the exposure solution for PAM production in the later stages of this study.

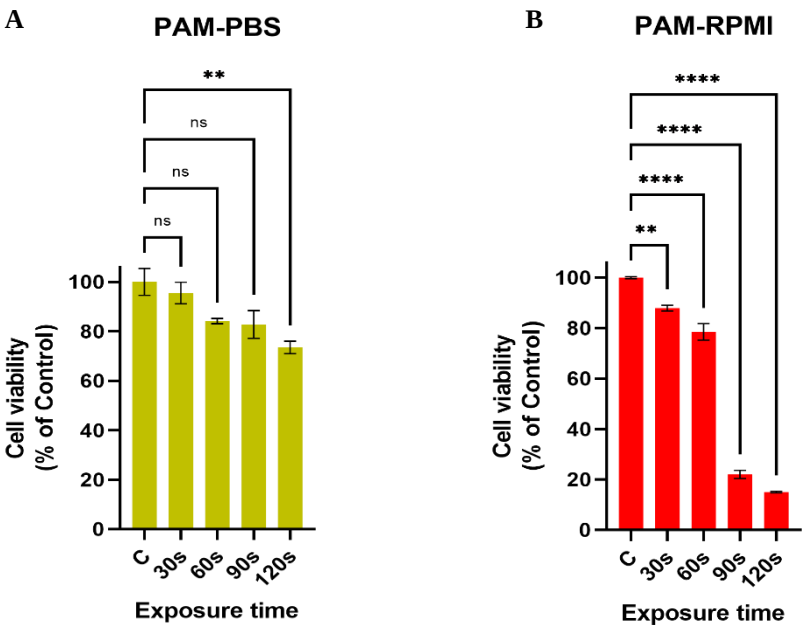

Figure 4.5. Viability of HOS cells treated with PAM-PBS (A) and PAM-RPMI (B). Values are mean±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, ** $p < 0.01$, **** $p < 0.0001$).

4.6. Temporal assessment of the effects of PAM-RPMI treatment on cell viability

Analyzing the evolution of the percentage of cell viability during the entire experiment, it was observed that all treatment options significantly reduce the number of viable cells from the first assessment, two hours after treatment. Even if in the case of certain experimental variants, at certain hours, no significant statistical differences were identified, the general trend observed was a progressive decrease in cell viability, this being most visible in the interval 12-48 hours post-treatment (Figure 4.6.). Thus we can state that after indirect PAM-RPMI treatment of HOS cells for 30 minutes, the effects of the treatment last up to at least 48 hours after its removal.

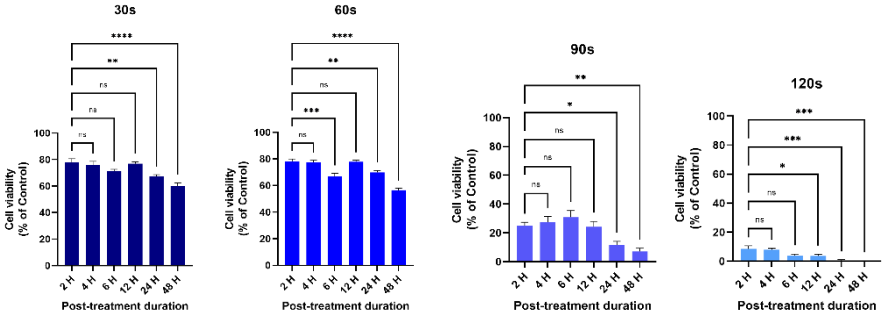

Figure 4.6. Viability of HOS cells treated with PAM-RPMI 30s (A), PAM-RPMI 60s (B), PAM-RPMI 90s (C) and PAM-RPMI 120s (D). Values are mean±SEM (n=8). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0004$ **** $p < 0.0001$)

4.7. Identifying the optimal treatment time

PAM-RPMI treatment showed cytotoxic effects for all experimental variants, ranging from mild to moderate and strong, depending on the combination of exposure time and treatment time (Figure 4.7).

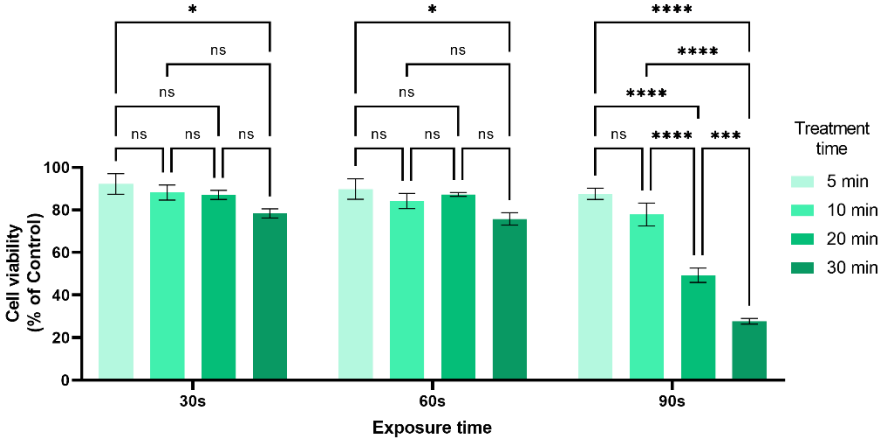

Figure 4.7. Comparative analysis of Ttr-influenced PAM-RPMI cytotoxicity. Values are mean±SEM (n=4). Statistical significance was determined by two-way ANOVA followed by Tuckey's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, *** $p = 0.0004$, **** $p < 0.0001$).

4.8. Establishing the influence of pH on cell viability

To investigate the impact of acidification of CAP-exposed medium on cells, two batches of HOS cells were used. One batch was treated with PAM-RPMI in three experimental variations (with Texp of 30, 60 and 90 seconds) and the other batch was treated with plain RPMI 1640 medium whose pH was adjusted with hydrochloric acid to reach pH values similar to those of PAM-RPMI.

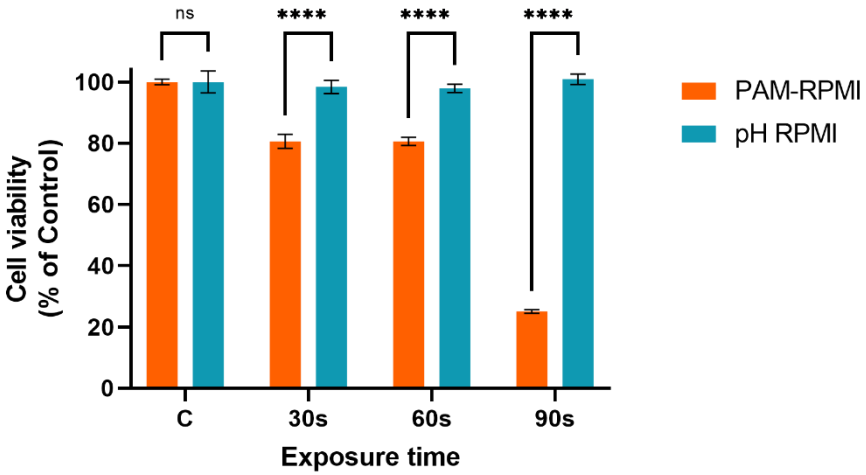

Figure 4.8. Viability of HOS cells treated with PAM-RPMI and pH-modified RPMI 1640 solution. Values are mean±SEM (n=4). Statistical significance was determined by two-factor ANOVA analysis, followed by Šídák post hoc multiple comparison test (ns – not significant, **** $p < 0.0001$).

Comparing the effects on the viability of HOS cells of the two types of treatments (pH-RPMI and PAM-RPMI), it was concluded that lowering the pH of RPMI 1640 does not influence the viability of HOS cells, the cytotoxic effects being therefore determined only by the exposure of RPMI 1640 to CAP.

4.9. Determination of treatment selectivity

The selectivity of indirect CAP treatment was evaluated by treating HOS tumor cells and normal HOB cells with PAM-RPMI and analyzing their viability at two predetermined time points, namely two and twenty-four hours post-treatment, respectively. The PAM-RPMI solutions used were exposed to CAP for 30, 60 and 90 seconds.

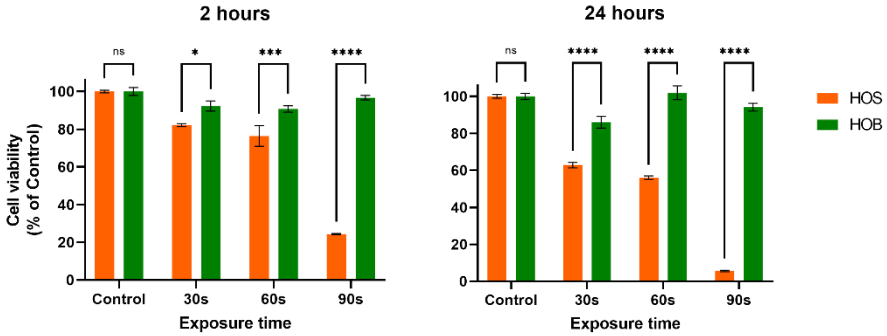

Figure 4.9. Evaluation of PAM-RPMI treatment selectivity. Values are mean±SEM (n=3). Statistical significance was determined by two-factor ANOVA followed by Šídák post hoc multiple comparison test (ns – not significant, * $p < 0.05$, *** $p = 0.001$, **** $p < 0.0001$).

After analyzing the results, it can be stated that the treatment with PAM-RPMI used in this study and produced by DBD-type discharge, presents a selective apoptotic effect in the case of osteosarcoma cells, with a maximum impact in the case of using a CAP exposure time of the RPMI 1640 medium of 90 seconds. Thus, the treatment has the ability to destroy HOS cells, with adjacent normal cells being affected to a very small extent.

4.10. Gene expression analysis

Following the analysis of the results obtained in the temporal evaluation of the effects of non-thermal plasma treatment at atmospheric pressure on HOS cells, it was

decided to carry out the gene expression analysis at two and twenty-four hours post-treatment.

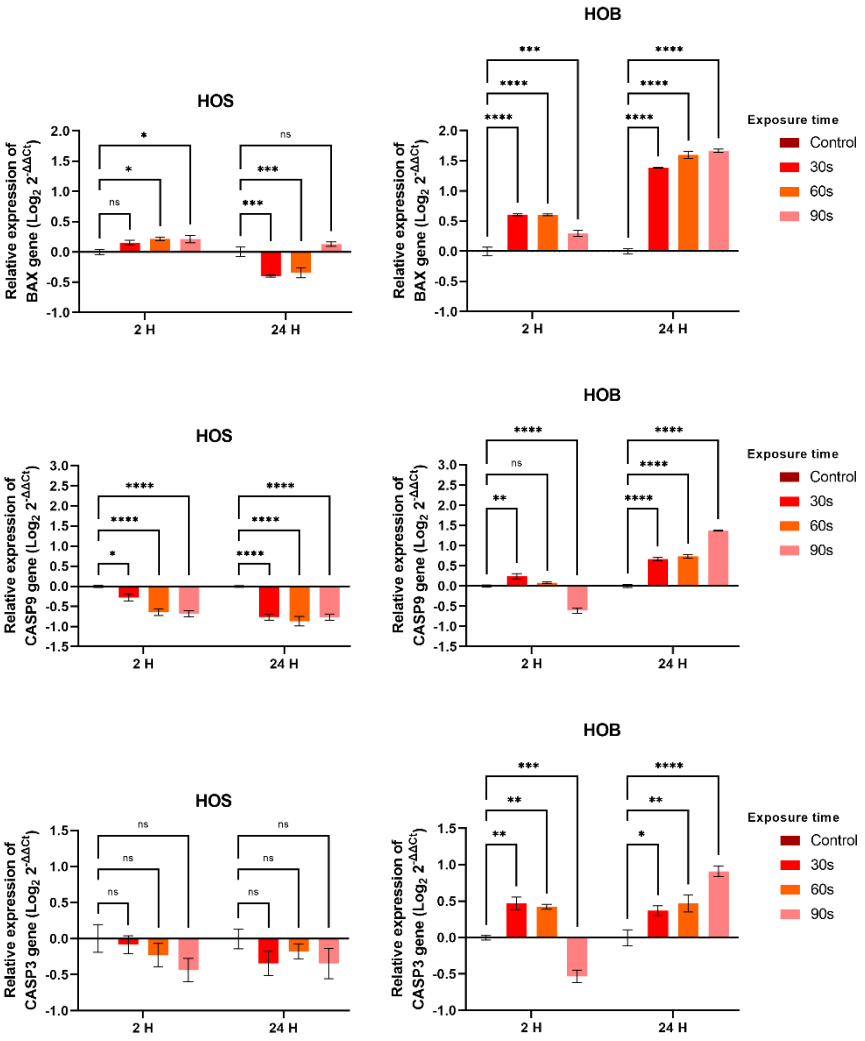

Figure 4.10. BAX, CASP9 and CASP3 gene expression analysis in HOS and HOB cells treated with PAM-RPMI. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

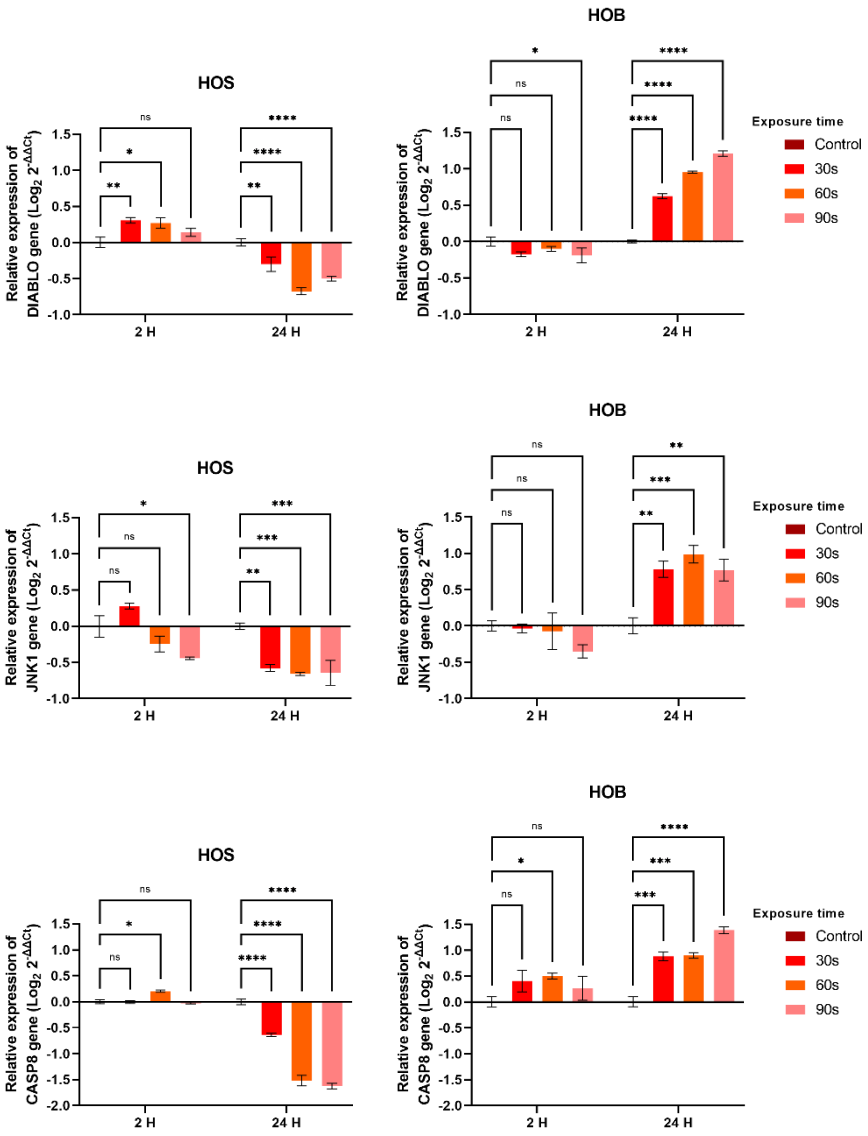

Figure 4.11. Expression analysis of DIABLO, JNK1 and CASP8 genes in HOS and HOB cells treated with PAM-RPMI. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

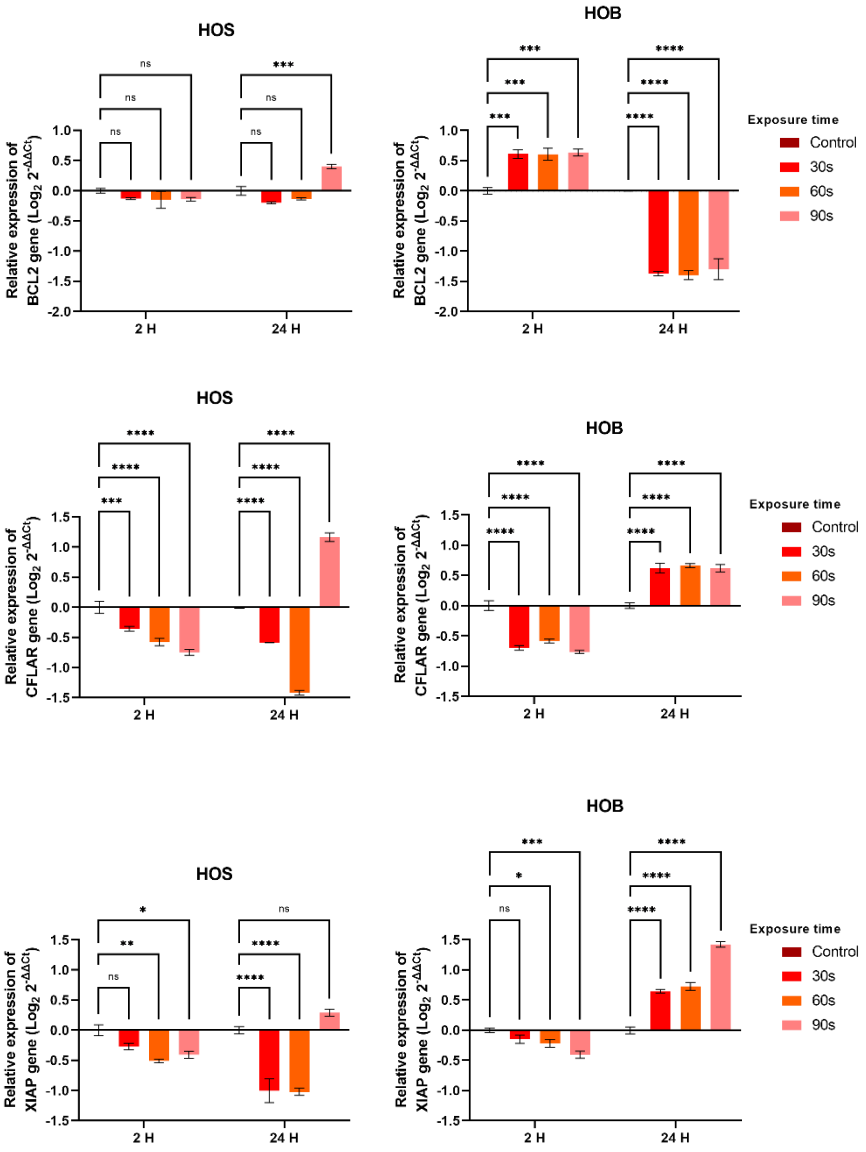

Figure 4.12. Expression analysis of BCL2, CFLAR AND XIAP genes in HOS and HOB cells treated with PAM-RPMI. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

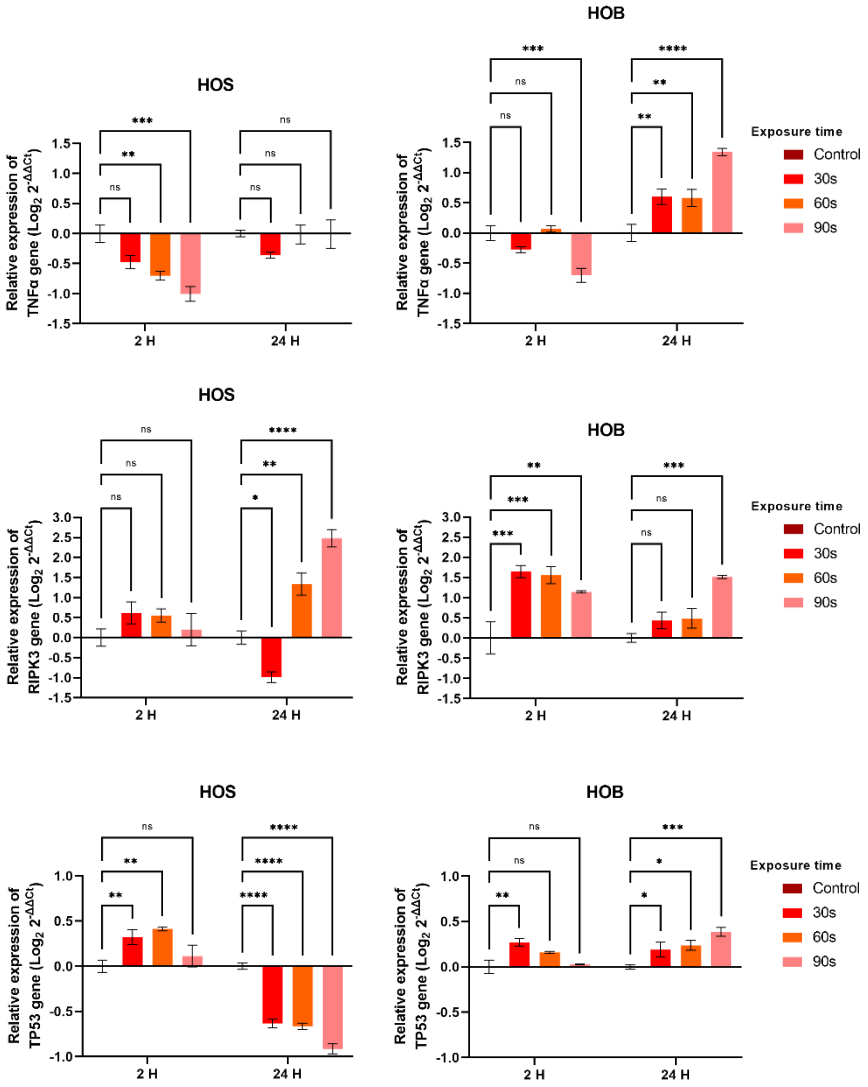

Figure 4.13. TNFα, RIPK3 and TP53 gene expression analysis in HOS and HOB cells treated with PAM-RPMI. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

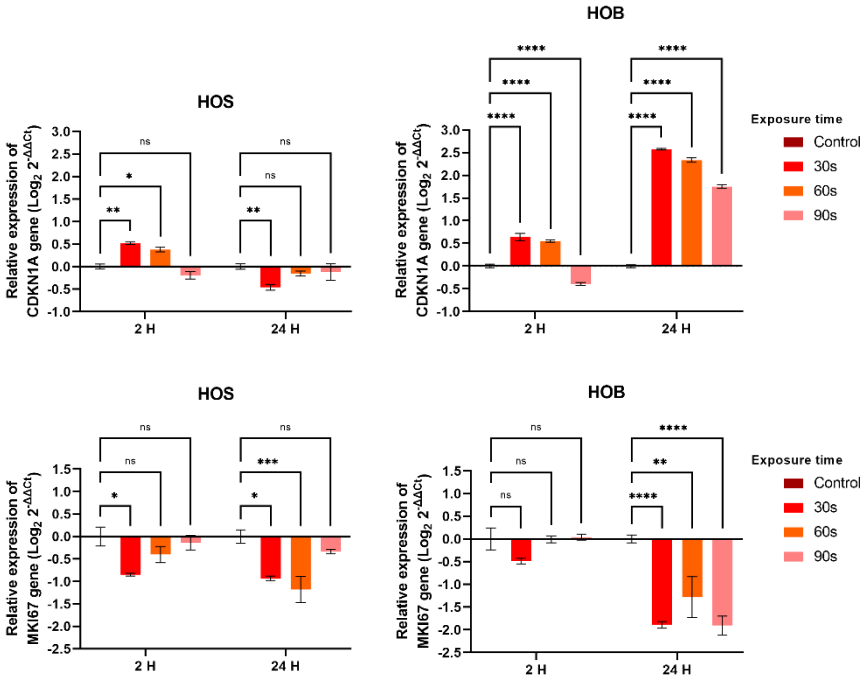

Figure 4.14. CDKN1A and MKI67 gene expression analysis in HOS and HOB cells treated with PAM-RPMI. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The first important aspect that stands out following the evaluation of gene expression in HOS and HOB cells treated with PAM-RPMI is the lack of dose-effect relationship (Figures 4.10 – 4.14). With a few exceptions, no correlation between exposure time and gene expression level could be identified, regardless of the post-treatment time point at which the analysis was performed. In contrast, similar changes in gene expression levels could be observed in samples treated with PAM-RPMI 30s and PAM-RPMI 60s, but different from the gene expression level of samples treated with PAM-RPMI 90s. Moreover, in the case of the evaluation of the expression level of some genes, the experimental treatment variants showed opposite modulations. Even if

this fact can be considered an impediment with regard to CAP therapy, the different modulation of the expression of some genes depending on the exposure time may represent a clinical advantage by being able to offer patients personalized treatments when it is desired to activate certain cell signaling pathways or, on the contrary, to avoid their activation.

Analyzing in general the effect of indirect treatment with CAP on the modulation of gene expression (Figures 4.10 – 4.14), not taking into account the result of each individual experimental variant, but the observed trend, it can be stated that, two hours after the treatment, PAM-RPMI had the effect of blocking the cell cycle and activating the intrinsic apoptosis signaling pathway in HOS cells through the overexpression of CDKN1A, TP53, BAX and DIABLO genes. According to this cellular signaling pathway, following the activation of the TP53 gene by various stimuli (including ROS), it activates the CDKN1A gene, which leads to cell cycle arrest, and the BAX gene, which in turn activates the DIABLO gene and releases cytochrome C. The DIABLO gene inhibits the expression of the antiapoptotic gene XIAP and, together with the BAX gene, activates the cascade of apoptotic caspases. Twenty-four hours after the treatment, the overexpression of the RIPK3 gene, involved in the process of cell death by necroptosis, was observed, while no overexpression of the genes involved in the apoptotic process was identified. Surprisingly, regardless of the time at which the assessments took place, no effect of activation of antiapoptotic signaling pathways was observed (except for PAM-RPMI 90s treatment, where, observing the cell viability data, the overexpression of these genes was late). Regarding the effects of indirect CAP treatment on HOB cells, it caused cell cycle arrest and activation of the intrinsic apoptosis signaling pathway through the overexpression of CDKN1A, TP53, BAX, CASP9 and CASP3 genes, activation of the extrinsic apoptosis signaling pathway through the activation of the CASP8 gene, as well as overexpression of the cellular necroptosis marker RIPK3. According to the intrinsic apoptosis signaling pathway, the activation of the TP53 gene leads to the activation of the CDKN1A gene, which blocks the cell cycle, and to the activation of the BAX gene, which releases cytochrome C. The

latter promotes the formation of the apoptosome, which results in the activation of the CASP9 gene, which, in turn, activates the CASP3 gene. Twenty-four hours after PAM-RPMI treatment, all genes that promote cell cycle arrest or cell death were overexpressed. Regarding the modulation of antiapoptotic gene expression in HOB cells by PAM-RPMI, overexpression of the BCL2 gene is identified two hours after treatment. It has the ability to inhibit apoptotic processes by inactivating the BAX and DIABLO genes. Twenty-four hours after the treatment, the overexpression of CFLAR genes, which has the role of inactivating CASP8 gene expression, and XIAP, which has the function of inactivating DIABLO, CASP9 and CASP3 genes, was observed. The underexpression of the MKI67 gene, in the case of both cell lines, both at two and twenty-four hours post-treatment, reveals that cell proliferation processes are suppressed.

The most important difference between the effects of PAM-RPMI on the gene expression of HOS and HOB cells is the overexpression of antiapoptotic genes in normal cells. This cellular response may underlie the selectivity of indirect CAP treatment, by blocking the proapoptotic signals induced by CAP in normal cells.

4.11. Determination of reactive nitrogen and oxygen species

4.11.1 RNS

Following the exposure of the RPMI medium to CAP discharges for 30 s, 60 s and 90 s, millimolar concentrations of NO_2^- and NO_3^- were identified in all experimental variants, these being generated in a CAP dose-dependent manner (Figure 4.15).

Analyzing the intracellular RNS levels, it was observed in both cell lines that they increase in a CAP dose-dependent manner immediately after treatment, followed by two hours to return to baseline values that are maintained up to twenty-four hours post-treatment (Figure 4.16). Interestingly, much higher concentrations of RNS were identified in HOB cells, in contrast to HOS cells.

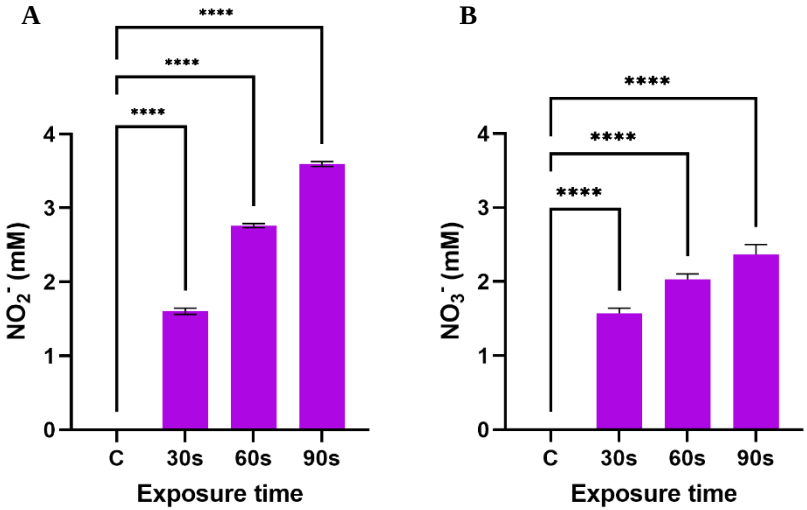

Figure 4.15. Quantification of NO_2^- (A) and NO_3^- (B) from PAM-RPMI solutions. Values are mean±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (**** p<0.0001)

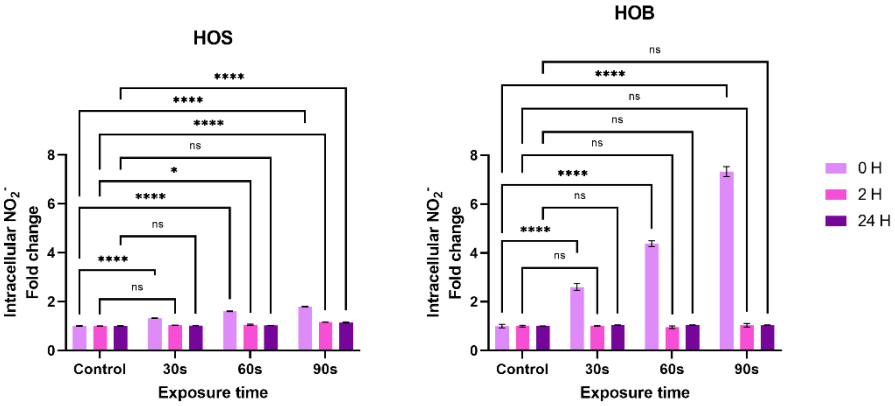

Figure 4.16. Evaluation of NO_2^- levels in HOS and HOB cells treated with PAM-RPMI. Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns - not significant, * p < 0.05, **** p < 0.0001)

4.11.2. ROS

Intracellular analysis of the level of ROS in tumor and normal cells, over a period of twenty-four hours after treatment (Figure 4.17) revealed that, in the case of HOB cells, immediately after treatment, the level of ROS decreased in a treatment dose-dependent manner. After two hours, values returned to normal, remaining constant up to twenty-four hours post-treatment. This reduction could result from an intense and immediate activation following PAM-RPMI treatment of cellular antioxidant mechanisms, their activity decreasing ROS concentrations below baseline. As for intracellular ROS levels in HOS cells, they underwent a slight increase (except for samples treated with PAM-RPMI 90s) and continued throughout the twenty-four hours. The accumulation of intracellular ROS that occurred after the removal of PAM treatments demonstrates an effect of CAP to induce the production of intracellular ROS or to deregulate antioxidant mechanisms, thus it can be stated that the biological effects are not based exclusively on the concentrations of reactive species generated by CAP and present in CAP.

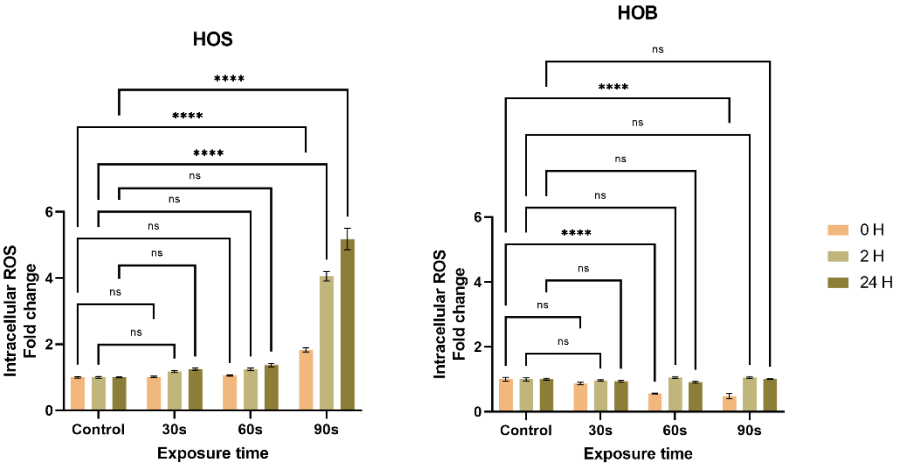

Figure 4.17. Analysis of ROS from PAM-RPMI-treated HOS and HOB cells. Values are mean ± SEM (n=6). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns - not significant, **** $p < 0.0001$)

General conclusions

Considering the experimental results of the present study, the following conclusions were drawn:

1. The electrical characterization of the CAP discharges revealed a typical current-voltage waveform, the dielectric barrier discharges being in the form of packets with a frequency of 100 Hz, and the frequency of the electrical voltage in each packet being 23 kHz.
2. The average number of currents within each discharge package is about 1600, with intensity values between 0.06 A and 0.72 A.
3. The CAP generating source produces PAM whose temperature during exposure does not exceed 30°C.
4. Upon exposure to CAP, PBS undergoes a more pronounced acidification process than RPMI 1640.
5. The optimal storage temperature of PAM solutions, without changing their pH levels, for a period of up to 3 months is -80°C, the most stable solutions being those of PAM-RPMI.
6. The CAP generation system produces PAM with cytotoxic effects on human osteosarcoma cells, dependent on the treatment dose.
7. PAM-RPMI has the ability to produce stronger cytotoxic effects on HOS cells than PAM-PBS.
8. Of all the experimental variants analyzed, PAM-RPMI treatment exposed to CAP for 120s demonstrates the strongest antitumor action, but could not be used in the evaluations of gene expression and RONS levels because the rate of cell death induced by it was almost 100%.
9. The cytotoxic effects of PAM-RPMI treatment are accentuated for at least forty-eight hours after its removal.
10. In the PAM-RPMI generation configuration shown, the optimal treatment time with the highest efficiency is 30 minutes.

11. The observed acidification of PAM-RPMI treatments does not influence cell viability, the cytotoxic effects being determined exclusively by the antitumor characteristics of CAP.
12. The CAP generation system used has the ability to produce PAM-RPMI with selective cytotoxic effects.
13. Modulation of gene expression following PAM-RPMI treatment is not dependent on treatment dose.
14. PAM-RPMI treatment of HOS cells caused cell cycle arrest and activation of apoptotic and necroptotic cell signaling mechanisms, by upregulating CDKN1A, TP53, BAX, DIABLO and RIPK3 gene expression.
15. PAM-RPMI treatment of HOB cells caused cell cycle arrest and activation of apoptotic and necroptotic cell signaling mechanisms by upregulating CDKN1A, TP53, BAX, DIABLO, JNK1, CASP9, CASP8, CASP3, TNFα and RIPK3 genes.
16. As a result of PAM-RPMI treatment, antiapoptotic cell signaling mechanisms are activated at the level of HOB cells, through the overexpression of BCL2, CFLAR and XIAP genes.
17. PAM-RPMI treatment results in both studied cell lines reducing or inhibiting cell proliferation processes by modulating the expression level of the MKI67 gene.
18. Activation of antiapoptotic signaling pathways in HOB cells may represent one of the mechanisms underlying the selectivity of indirect CAP treatment.
19. Exposure of RPMI 1640 medium to CAP discharges generated by the system presented in this study produces the highest levels of RNS reported to date for the effects of indirect CAP treatments in oncology.
20. PAM-RPMI treatments dose-dependently increase intracellular NO_2^- levels in both cell types evaluated in this study.
21. PAM-RPMI treatments cause intracellular ROS accumulation in HOS cells in a dose-dependent manner, with HOB cells having the ability to keep intracellular ROS levels under control.

22. According to the obtained results, the degree of cytotoxicity of PAM-RPMI treatment is dictated by its potential to induce changes in intracellular ROS concentrations, the ability of HOB cells to counteract oxidative stress conferring resistance to CAP treatment.
23. Considering the obtained experimental results, PAM-RPMI solutions obtained with the presented CAP generation system could represent a promising approach in the therapy of osteosarcoma.

Published articles

List of papers published as the first author

1. **Stache, A.B**.; Mihăilă, I.; Gerber, I.C.; Dragoș, L.M.; Mihai, C.T.; Ivanov, I.C.; Topală, I.; Gorgan, D.-L., 2023. Optimization of Indirect CAP Exposure as an Effective Osteosarcoma Cells Treatment with Cytotoxic Effects. Appl. Sci., 13, 7803. https://doi.org/10.3390/app13137803
2. **Stache, A.B**.; Mihăilă, I.; Gerber, I.C.; Dragoș, L.M.; Mihai, C.T.; Ivanov, I.C.; Topală, I.; Gorgan, D.-L., 2023. Cold atmospheric plasma treatment modulates the expression of cdk1, tnfα and tp53 genes in human osteosarcoma cells. J Exp Molec Biol 24(3):x-xx. https://doi.org/10.47743/jemb-2023-100

List of papers published as co-author

1. Dragoș, M.L.; Ivanov, I.C.; Mențel, M.; Văcărean-Trandafir, I.C.; Sireteanu, A.; Titianu, A.A.; Dăscălescu, A.S.; **Stache, A.B.**; Jitaru, D.; Gorgan, D.L., 2022. Prognostic Value of Association of Copy Number Alterations and Cell-Surface Expression Markers in Newly Diagnosed Multiple Myeloma Patients. Int. J. Mol. Sci., 23, 7530. https://doi.org/10.3390/ijms23147530

2. Boiangiu, R.S.; Mihasan, M.; Gorgan, D.L.; **Stache, B.A.**; Hritcu, L., 2021. Anxiolytic, Promnesic, Anti-Acetylcholinesterase and Antioxidant Effects of Cotinine and 6-Hydroxy-L-Nicotine in Scopolamine-Induced Zebrafish (Danio rerio) Model of Alzheimer's Disease. Antioxidants, 10, 212. https://doi.org/10.3390/antiox10020212
3. Boiangiu, R.S.; Mihasan, M.; Gorgan, D.L.; **Stache, B.A.**; Petre, B.A.; Hritcu, L., 2020. Cotinine and 6-Hydroxy-L-Nicotine Reverses Memory Deficits and Reduces Oxidative Stress in Aβ25-35-Induced Rat Model of Alzheimer's Disease. Antioxidants, 9, 768. https://doi.org/10.3390/antiox9080768

Participation in scientific events

List of presentations and posters presented at international conferences

1. **Stache A.B.**, Dragoș M.L., Ivanov I.C., Mihăilă I., Topală I., Mihai C.T., Gorgan D.L., The impact of two different cold atmospheric plasma-exposed media treatments on the osteosarcoma cells viability. *CONFER 2020*, November 19th-22nd, 2020, Iași, România.
2. **Stache A.B.**, Dragoș M.L., Ivanov I.C., Topală I., Mihăilă I., Mihai C.T., Gorgan D.L., Cold atmospheric plasma indirect treatment generates different anti-apoptotic genetic response in osteoblasts and osteosarcoma cells. *CONFER 2021*, November 18th-21st, 2021, Iași, România.
3. **Stache A.B.**, Dragoș M.L., Ivanov I.C., Topală I., Mihăilă I., Mihai C.T., Gorgan D.L., Evaluation of the intracellular reactive oxygen and nitrogen species induced by cold atmospheric plasma treatment. *CONFER 2022*, November 24th-26th, 2022, Iași, România.
4. Gherghel D., Cădinoiu A.N., Atanase L.I., Popa M., Sande A.S., Mihai C.T., **Stache A.B**., Vochița G., In vitro biocompatibility assessment of new functionalized chitosan-coated liposome nanoparticles. *XXXI th edition of the*

International Congress of "Apollonia" University of Iasi "By promoting excellence, we prepare the future", March 2nd-5th, 2023, Iași, România.

5. Brînza I., **Stache A. B.**, Omayma E., Gorgan D.L., Mihășan M., Hrițcu L., Sweroside supports memory function by increasing mRNA expression of bdnf, creb, npy and decreasing AChE activity and brain oxidative stress in the scopolamine induced zebrafish model (*Danio rerio*). *YSF congresis*. July 6th-9th, 2022, Vimeiro, Portugal,.
6. Brînza I., **Stache A. B.**, Omayma E., Gorgan D.L., Mihășan M., Hrițcu L., Sweroside supports memory function by increasing mRNA expression of bdnf, creb, npy and decreasing AChE activity and brain oxidative stress in the scopolamine induced zebrafish model (*Danio rerio*). *IUBMB-FEBS-PABMB congresis*, July 9th-14th , 2022, Lisbon, Portugal.
7. Brînza I., **Stache A. B.**, Omayma E., Gorgan D.L., Mihășan M., Hrițcu L., Baicalein Prevent Scopolamine-Induced Memory Impairment in Zebra Fish by Increasing the Creb Protein Level and the mRNA Expression Of Bdnf and Creb1 Gene. *„Life sciences in the dialogue of generations: connections between universities, academia and business community"*, September 29th-30th, 2022, Chisinau, Republica Moldova.
8. Brînza I., **Stache A. B.**, Omayma E., Gorgan D.L., Mihășan M., Hrițcu L., Sweroside supports memory function in the scopolamine-induced zebrafish model (*Danio rerio*) of Alzheimer disease, International conference and XXXIXth scientific session of the Romanian Society for Cell Biology, 21st-23rd, 2022, Cluj-Napoca, Romania.

List of presentations and posters presented at national conferences

1. **Stache A.B.**, Dragoș M.L., Mihăilă I., Topală I., Gorgan D.L., Cold plasma-induced apoptosis in osteosarcoma cells. *11th national congress with international participation and 37th annual scientific session of Romanian Society for Cell Biology*, June 20th-23rd, 2019, Constanța, România.

2. **Stache A.B.**, Dragoș M.L., Mihăilă I., Topală I., Gorgan D.L., CAP treatment modulates the apoptosis and cell cycle control in a human osteosarcoma cell line. *Conferința școlilor doctorale din cadrul Universității „Alexandru Ioan Cuza" din Iași*, October 22nd-23rd, 2020, Iași, România.
3. **Stache A.B.**, Dragoș M.L., Topală I., Mihăilă I., Mihai C.T., Gorgan D.L., Impactul tratamentului cu plasmă non-termică la presiune atmosferică asupra expresiei genelor asociate controlului apoptozei. *Sesiunea științifică a Facultății de Biologie - Tendințe în biologie: de la molecule la sisteme complexe*. October 28th-29th, 2021, Iași, România.

Research projects - member

1. Member of the target group of the project *Creșterea numărului absolvenților de învățământ terțiar universitar și non universitar care își găsesc un loc de muncă urmare a accesului la activități de învățare la un potențial loc de muncă / cercetare/ inovare, cu accent pe sectoarele economice cu potențial competitiv identificate conform SNC și domeniile de specializare inteligentă conform SNCDI*. POCU/380/6/13/123623.
2. Scientific research assistant within the project *Active targeted drug delivery systems based on peptide-functionalized magnetic nanoparticles for the treatment of inner ear diseases (TargEar)*. RO-NO-2019-0187.
3. Scientific research assistant within the project *Dezvoltarea Stației de monitorizare a peștilor migratori: sturioni și scrumbie – Isaccea*. Cod SMIS 2014+ 136849.

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