COLD ATMOSPHERIC PLASMA TREATMENT MODULATES THE EXPRESSION OF *cdk1*, *tnf α* AND *tp53* GENES IN HUMAN OSTEOSARCOMA CELLS

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Abstract

Osteosarcoma (OS), a malignancy primarily affecting children and adolescents, is the most frequently encountered malignant, non-hematologic, bone tumor. Despite the gradual improvement of survival rates, the management of this disease remains problematic due to challenges such metastasis development, its heterogeneous characteristics, and resistance to cytostatic drugs. Cold atmospheric plasma (CAP), a partially ionized gas operating at near room temperature, which is comprised of free carriers, excited or neutral molecules, and active radicals capable of initiating diverse physical phenomena and chemical reactions represent a new and innovative potential solution in cancer therapy. The aim of this study was to evaluate the capacity of CAP produced by our custom-build plasma source to induce cytotoxic effects to HOS cells and to analyze post-treatment modulations of *cdk1*, *tnf α* and *tp53* genes expression. Direct and indirect CAP treatments effectiveness on HOS cells were evaluated by MTT assay and the regulation of interest genes expression were carried out by RT-qPCR analysis. Cell viability analysis revealed a strong cytotoxic effect of direct CAP treatment on HOS cells, while the indirect treatment resulted in a slight decrease of cells viability. Direct CAP treatment modulates the expression of all analyzed genes, both at 2- and 24-hours post-treatment. In conclusion, direct CAP treatment produced by our custom-build plasma source have cytotoxic effects on HOS cells up to 24 hours post-treatment. Furthermore, direct CAP treatment induces cell cycle arrest of HOS cells, and the CAP-induced cell death is independent of *tp53* gene.

Keywords: cold atmospheric plasma, osteosarcoma, *cdk1*, *tnf α*, *tp53*.

Received 30 May 2023; Accepted 17 July 2023; Published Online 17 July 2023; Published xxxx

Introduction

Osteosarcoma (OS) is a primary bone cancer that predominantly affects children and young adults. This type of cancer has mesenchymal origins and usually develops around the metaphysis of long bones, producing immature bone tissue (Lindsey et al. 2016; Eaton et al. 2021). Although the survival rate for osteosarcoma has increased from below 30% to approximately 70% over time, the treatment is still challenging due to metastasis, its heterogeneous nature, and resistance to cytostatic drugs (Misaghi et al. 2018; Yang et al. 2020; Sheng et al. 2021). The current treatment consists of surgical intervention combined with neoadjuvant and post-operative chemotherapy, using mainly cisplatin, doxorubicin, methotrexate and isophosphamide (Lindsey et al. 2016). Due to the risk of local recurrence, tumor resections must be performed with negative margins, otherwise the affected limb may be amputated (Eaton et al. 2021). Although this radical intervention can result in a significant increase in the survival prognosis, a decrease in the patient's quality of life in terms of social relationships has been reported (Eaton et al. 2021). The recurrence rate, as well as the poorer prognosis that accompanies it, are mainly defined by the microlesions around the tumor and the microresidual tumors that cannot be completely removed by surgery. Therefore, in order to overcome these challenges, as well as better manage the metastatic nature of osteosarcoma and its chemoresistance, new alternative or complementary treatment methods are needed in addition to the current ones (Gümbel, Suchy, et al. 2017; Xu et al. 2020). Cold atmospheric plasma (CAP), a partially ionized gas consisting in ions, free electrons, molecules, photons, free radicals, and a large specter of reactive species, represent a promising new alternative in cancer therapy (Yan et al. 2017; Baik et al. 2023; Cui et al. 2023). An increasing number of studies demonstrate the potential of CAP treatment in cancer therapy. Thus, by studying various cancer types, diverse advantageous outcomes of CAP treatment have been identified, including selective cytotoxicity, modifications in the tumor microenvironment, sensitization of tumor cells, enhancement of nanoparticles effects, improvements in the antitumor effectiveness of cytostatics and the overcoming of drug resistance (Zhu et al. 2016; Dai et al. 2020; Yan et al. 2021; Mateu-Sanz et al. 2022; Min et al. 2022; Murillo et al. 2023). In biomedical studies, two main methods of CAP treatment are widely used: direct treatment, where the target to be treated is subjected to CAP discharges, and indirect treatment, where a solution is first exposed to CAP and then transferred to the target (Malyavko et al. 2020). Although Saadati et al. asserted that the location of the tumor is an important factor in deciding the best treatment approach and direct exposure may not be feasible for tumors located inside the body (Saadati et al. 2018), Malyavko et al. proposed direct treatment as a substitute for surgical intervention or a complementary method to it, and indirect treatment as a pharmacological procedure (Malyavko et al. 2020). Until now, the best treatment method between the two has not been clearly identified. Depending on the type of cancer, the type of study, the cell culture media used, the molecular mechanisms or the reactive species monitored, these two treatment methods alternate in terms of efficiency and effectiveness. *In situ* and *in vivo* experiments conducted by Zhou et al. demonstrated that, although similar efficiencies were recorded in terms of reducing cancer cell proliferation and migration, recovering of white blood cell indexes, and regulating renal dysfunction, higher survival rates of treated mice, along with reduced costs and improved safety, promote direct treatment as the better method (Zhou et al. 2020). Analyzing the influence of cancer type and cell culture media on the effects of direct and indirect treatments, Biscop et al. demonstrated that variation in these parameters strongly influence the indirect treatment compared to the direct one (Biscop et al. 2019). Malyavko et al. demonstrated that although reactive oxygen and nitrogen species (RONS) underlie the antitumor effects of both indirect and direct treatment, the ability of the latter to induce CAP-treated cells into an activated state makes them more vulnerable to RONS, thereby making the direct treatment

method superior to the indirect one (Malyavko et al. 2020). In a study conducted by Saadati et al., even though the direct treatment demonstrated a more pronounced cytotoxic effect on tumor cells than the indirect treatment, the latter presented less toxic side effects. Additionally, by combining the indirect treatment with chemotherapy, the most effective method of antitumor treatment was obtained (Saadati et al. 2018). Similarly, in a study comparing the effects of both types of CAP treatments on normal bone marrow mesenchymal stem cells (BMMSCs), Hajizadeh et al. demonstrated the superiority of the indirect method due to the reduced cellular damage it caused (Hajizadeh et al. 2019). Thus, it can be observed that there is still an uncertainty regarding the selection of one of the methods as being superior to another. While direct treatment is the most commonly used method in studies exploring the effects of CAP in cancer therapy, there has been a continuous increase in the number of studies using indirect treatment (Dubuc et al. 2018). Regarding the evaluation of the effects of CAP treatment on osteosarcoma, it appears that the field of interest is still in its early stages. In 2018, Dubuc et al. presented in a systematic review that out of a total of 190 articles, only 6 focused on osteosarcoma (Dubuc et al. 2018). By analyzing articles on the Web of Science core collection, it can be observed that there are only 34 articles specifically dedicated to the study of CAP treatment for osteosarcoma. Furthermore, although there is constant progress in this field, the mechanisms of action as well as the signaling pathways activated by CAP treatment are not fully known or understood and require further studies to lead the use of CAP as a therapeutic method against cancer (Haralambiev et al. 2019; Hasse et al. 2019; Malyavko et al. 2020; Murillo et al. 2023). Considering the aspects that have been mentioned earlier, the aim of this study is to assess the ability of CAP produced by our custom-made source to induce cytotoxic effects on osteosarcoma cells, using both direct and indirect treatment methods, and to investigate the impact of CAP treatment on the expression levels of specific genes involved in cell cycle regulation and cell death.

Materials and Methods

Cell culture. Human osteosarcoma cells (HOS) (CRL-1543, American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Sigma Aldrich, MO, USA) supplemented with fetal bovine serum 10% (Sigma Aldrich, MO, USA) and penicillin-streptomycin antibiotics 1% (Biological Industries, Kibbutz Beit-Haemek, Israel). Cells were maintained and expanded at 37°C, 5% CO_2 and 95% humidity in a Certomat CO_2 Incubator (Sartorius Stedim Biotech GmbH, Göttingen, Germany).

CAP treatment. In order to produce CAP, a custom-made dielectric barrier discharge without flow system was used. CAP discharges took place in a polypropylene 24-well cell culture plate (TPP Techno Plastic Products AG, Trasadingen, Switzerland), between a high voltage electrode made of 4 cylindrical stainless-steel subunits and a ground electrode represented by a copper plate placed under the culture plate. Between the bottom of the culture plate and the ground electrode was positioned a 0.1 mm dielectric layer. The system was powered up by an AC power supply with maximum voltage of 15 kV and a sinusoidal waveform. Due to the high-voltage electrode configuration, CAP exposures were carried out one after the other, exposing one column of the culture plate at a time. Thus, the discharge parameters were identical for all 4 wells of the column. In this study, in order to evaluate the biological effects of CAP treatment on HOS cells, three exposure times were used: 30 seconds, 60 seconds and 120 seconds. This represents the periods of time in which the source was turned on and the CAP discharges occurred.

Direct CAP treatment. Direct treatments were performed by exposing 5 x 10^5 HOS cells/well to CAP discharges, for previously presented periods of time. Depending on these exposure times, we will address samples directly treated as CAP 30s, CAP 60s and CAP 120s. Cells were seeded in the culture plate 1-day prior exposures, in 500 µl complete RPMI 1640

medium. Before treatments, culture medium was replaced with 150 µl Dulbecco's phosphate-buffered saline (PBS) (Biological Industries, Kibbutz Beit-Haemek, Israel). After treatment, PBS was removed and 500 µl complete RPMI 1640 medium per well was added.

Indirect CAP treatment. One day before treatment, 5 x 10^5 HOS cells were seeded in a 24-well cell culture plate, in 500 µl complete RPMI 1640 medium. For indirect treatment, 150 µl PBS was dispensed into each well of the discharge vessel and exposed to CAP for previously mentioned exposure times, thus obtaining plasma-activated medium (PAM). In this study, depending on the exposure time used, we will refer to the CAP exposed PBS for the indirect treatment as PAM-PBS 30s, PAM-PBS 60s and PAM-PBS 120s. After PAM generation, the medium from the seeded cells was discarded and, after a washing step with PBS, 150 µl of PAM/well were added. For each exposure time, four wells/plate were treated with PAM for three minutes. After treatment, PAM was replaced with 500 µl complete RPMI 1640 medium per well.

Cell viability assay. In order to evaluate the cytotoxic effects of CAP treatment, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed at 2- and 24-hours post-treatments. After this period of incubation, cell culture medium was discarded and the cells were washed with PBS. Subsequently, 550 µl of MTT working solution consisting of 500 µl RPMI 1640 and 50 µl MTT 5mg/ml were added in each well. After a two hours incubation step at 37°C, 5% CO_2 and 95% humidity, 450 µl of MTT working solution was discarded followed by the addition of 350 µl DMSO. Cells were incubated for 10 minutes, afterwards the absorbance was measured by using the multimodal plate reader FilterMax F5 (Molecular Devices LLC, CA, USA) at 570 nm wavelength.

Gene expression analysis. Total ARN was extracted at 2- and 24-hours post treatment using SV Total RNA Isolation System (Promega, WI, USA) following manufacturer's instructions and spectrophotometrically quantified by measuring its absorbance at 260 nm by NanoDrop 2000 Spectrophotometer (ThermoFisher, Waltham, MA, USA). The reverstranscription and real-time PCR amplification of *cdk1, cdk2, tnf α* and *tp53* genes was performed in a single step reaction by GoTaq® 1-Step RT-qPCR System (Promega, Wi, USA) on a Rotor Gene 6000 Q 5-plex HRM thermocycler (Corbett Research, QIAGEN Corporation, Germany). The primer sequences were CDK1 F 5'- GAATCCGGGGCCCTTTAGCG -3', CDK1 R 5'- ACAACAACCGCGTCGCTCTCC -3', TNF α F 5'- ATGAGCACTGAAAGCATGATCC -3', TNF α R 5'- GAGGGCTGATTAGAGAGAGGTC -3', TP53 F 5'- CAGCACATGACGGAGGTTGT -3', TP53 R 5'- TCATCCAAATACTCCACACGC -3'. In order to amplify and quantify the expression level of each targeted gene, the following thermal cycling parameters were used: 40°C for 15 minutes and 95°C for 10 minutes followed up by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. Data acquisition was performed with Rotor-Gene Q - Pure Detection Software v. 2.2.3. (QIAGEN Corporation, Germany). The gene expression modifications were evaluated by comparing the gene expression levels in CAP treated samples with the gene expression levels in untreated control samples and reported as a fold change. Each measurement was performed in triplicate.

Signaling pathways identification for simultaneous regulation of the analyzed genes expression. The evaluation of cellular signaling pathways was conducted using the GeneCards database – the human gene database (Stelzer et al. 2016), and the analysis platform The SIGnaling Network Open Resource (SIGNOR 3.0) (Lo Surdo et al. 2023). The analysis aimed to identify direct or indirect signaling pathways between the explored genes in this study, which exhibited a similar modulation of their expression levels. It consisted of the following steps: a. Investigation of direct signaling pathways. b. Investigation of indirect signaling pathways. c. Identification of regulators whose activity involves transcriptional regulation of the target

genes. d. Identification of signaling pathways connecting regulators identified in the previous step (referred in this study as interrelating regulators).

Results

Cell viability. In order to evaluate the response of osteosarcoma cells to treatment with CAP, in this study the viability of HOS cells treated with CAP was checked both by the direct method and by the indirect method. For this, three CAP exposure times were evaluated: 30 seconds, 60 seconds and 120 seconds. Control groups were used for each individual treatment method, the cells within these groups being manipulated identically to those treated with CAP, only that they were not subjected to the treatments. The results are expressed in percentages and represent the changes identified in relation to the values of the samples from the control groups. Two hours after the direct treatment, the cells showed a decrease in viability of down to 27.4% for the 30s exposure, 9.9% for the 60s exposure and 2.1% for the 120s exposure. According to Dunnett's post hoc test, all results are statistically significant. In the case of the indirect treatment, two hours post-treatment the viability of cells treated with PAM-PBS 30s was 103.3%, of those treated with PAM-PBS 60s was 91%, and of those treated with PAM-PBS 120s was 85.3%. The Dunnett post-hoc test reveals that only in the case of treatment with PAM-PBS 120s the change in viability is statistically significant (Figure 1).

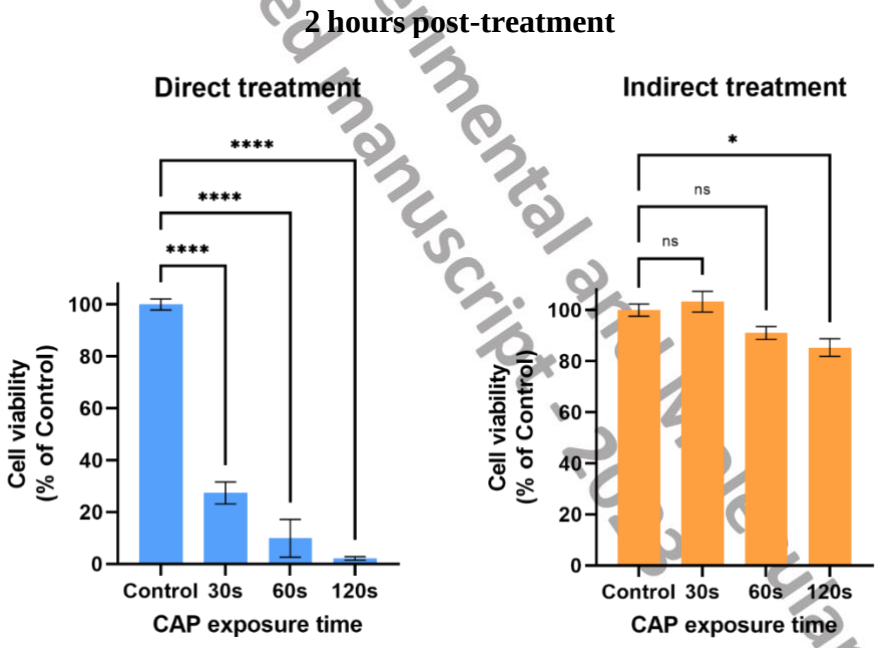

Figure 1. Viability of human osteosarcoma cells (HOS) treated with CAP by direct method (left) and indirect method (right) at 2 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, * $p<0.05$, **** $p<0.0001$).

Twenty-four hours after the treatment, the cells showed relatively the same trend of change in cell viability as after the evaluation two hours post-treatment. In the case of direct treatment, the viability of cells treated with CAP decreased to 51.2% for the 30s exposure, 6.7% for the 60s exposure and 13.4% for the 120s exposure. The indirect treatment had the effect of changing cell viability, as follows: 99.6% for PAM-PBS 30s, 96.3 for PAM-PBS 60s and 83%

for PAM-PBS 120s. According to the Dunnett post-hoc test, in the case of direct treatment all changes in cell viability were statistically significant, and in the case of indirect treatment only the observed effects of the treatment with PAM-PBS 120s were significant (Figure 2). Comparing the effects of the two treatment methods for each individual exposure time, according to the Šídák post-hoc test, there are statistically significant differences for all the experimental variants analyzed in this study, both at 2- and 24-hours post-exposure (Figure S1 and Figure S2).

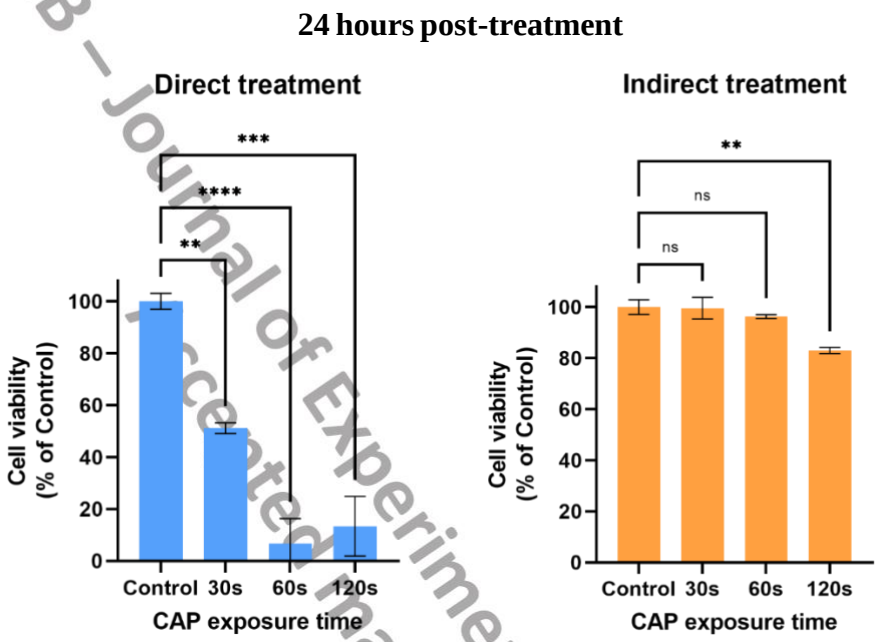

Figure 2. Viability of human osteosarcoma cells (HOS) treated with CAP by direct method (left) and indirect method (right) at 24 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, ** $p<0.01$, *** $p<0.005$, **** $p<0.0001$).

Gene expression.

***CDK1*.** Direct treatment with CAP on osteosarcoma cells resulted in a decrease in *cdk1* gene expression levels at two hours post-treatment. When compared to control samples, exposure to CAP for 30s, 60s, and 120s downregulated *cdk1* gene by 0.52-fold, by 0.77-fold, and by 0.29-fold, respectively. However, after 24 hours, all experimental variants showed an upregulation of the *cdk1* gene by 1.24-fold, by 2.61-fold, and by 3.18-fold for the 30s, 60s, and 120s exposures, respectively. According to the Dunnett post-hoc test, except for the change in the expression level of the *cdk1* gene evaluated 24 hours after treatment in cells exposed to CAP 30s, all other identified results were statistically significant (Figure 3).

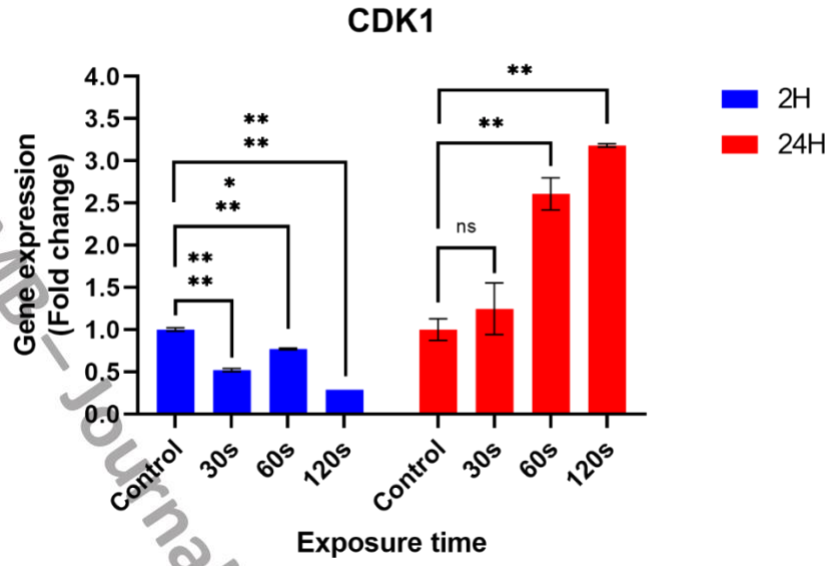

Figure 3. Analysis of *cdk1* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, ** p<0.01, *** p<0.005, **** p<0.0001).

TNF α. After treatment with CAP, the expression level of the *tnf α* gene in osteosarcoma cells was analyzed. Results showed that two hours after treatment, the expression level of *tnf α* decreased in all experimental variants compared to control samples (by 0.73-fold for CAP 30s, by 0.59-fold for CAP 60s, and by 0.68-fold for CAP 120s). However, twenty-four hours after treatment, the samples treated with CAP 30s presented a similar gene expression level to the control samples, while CAP 60s and CAP 120s treatments upregulated *tnf α* gene by 1.52-fold and by 1.32-fold, respectively. Despite that, according to the Dunnett post-hoc test, no change in the level of *tnf α* gene expression showed statistical significance (Figure 4).

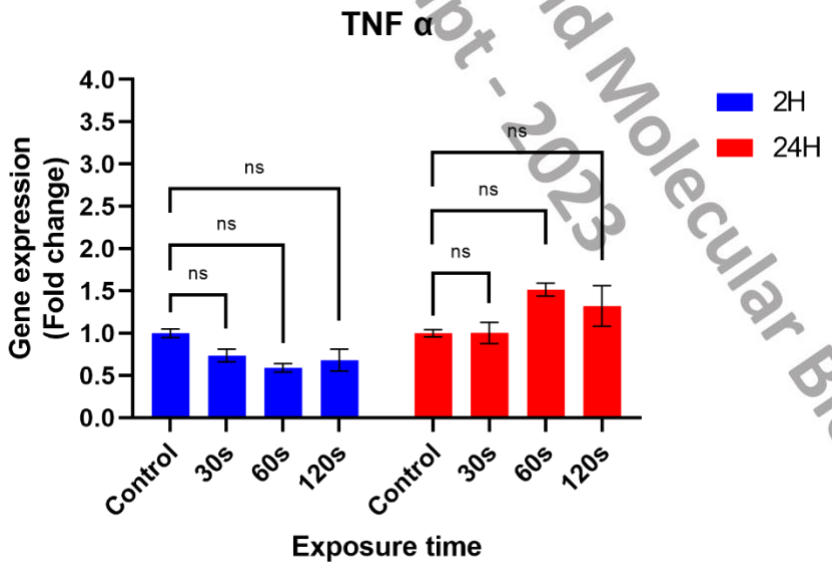

Figure 4. Analysis of *tnf α* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant).

TP53. Two hours after CAP 30s treatment, *tp53* gene was upregulated by 1.47-fold. In contrast, the 60s and 120s treatments resulted in its downregulation by 0.91-fold and by 0.1-fold, respectively). The evaluation of the *tp53* gene expression twenty-four hours after the treatment revealed its downregulation for all experimental variants, as follows: by 0.78-fold for CAP 30s, by 0.16-fold for CAP 60s, and by 0.08-fold for CAP 120s. The Dunnett post-hoc test shows that all identified changes in the expression levels of the *tp53* gene are statistically significant (Figure 5).

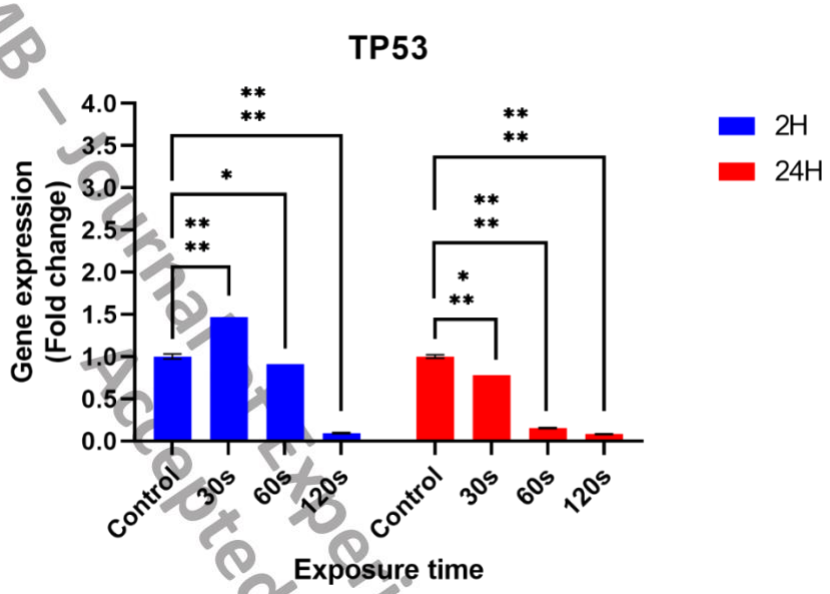

Figure 5. Analysis of *tp53* gene expression in CAP-treated HOS cells at 2- and 24-hours post-treatment. Values are mean ± SEM (n=3). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc multiple comparison test (ns – nonsignificant, * p<0.05, *** p<0.005, **** p<0.0001).

Identification of the signaling pathways linkage. At 2 hours post-treatment both *cdk1* and *tnf α* genes were downregulated. According to SIGNOR tool the *cdk1* gene is downregulated by KDM2B and Noncanonical PRC1, and *tnf α* gene is downregulated by MCR1, KLF4, MFGE8 and FUBP1. Evaluating the signaling linkage between the identified regulators of both genes, we have identified two interrelating regulators, MCR1 and KLF4 (Figure 6).

Figure 6. Evaluation of signaling pathways linkage between *cdk1* and *tnf α* resulting in downregulation of both genes (---▸ indirect upregulation, ---┤ indirect downregulation).

At 24 hours post-treatment, *cdk1* and *tnf α* genes were upregulated, while *tp53* was downregulated. Using the SIGNOR tool, we have evaluated the possible signaling pathways which modulates the expression of our interest genes as previously presented.

The *cdk1* gene is upregulated by E2F1 and TFDP1, *tnf α* gene is upregulated by A9/b1 integrin, NfKb-p65/p50, RBM10, HBA1, IRF5, ANXA1, HBB and IL1A, and *tp53* gene is downregulated by KLF4, DRAM2, CBFbeta-MYH11 and U2AF1. Firstly, in order to evaluate common signaling pathways whose activity have as result modulation of *cdk1*, *tnf α* and *tp53* genes in the same manner as we observe following RT-qPCR post-CAP treatment analysis, we evaluated the interrelating regulators between each gene.

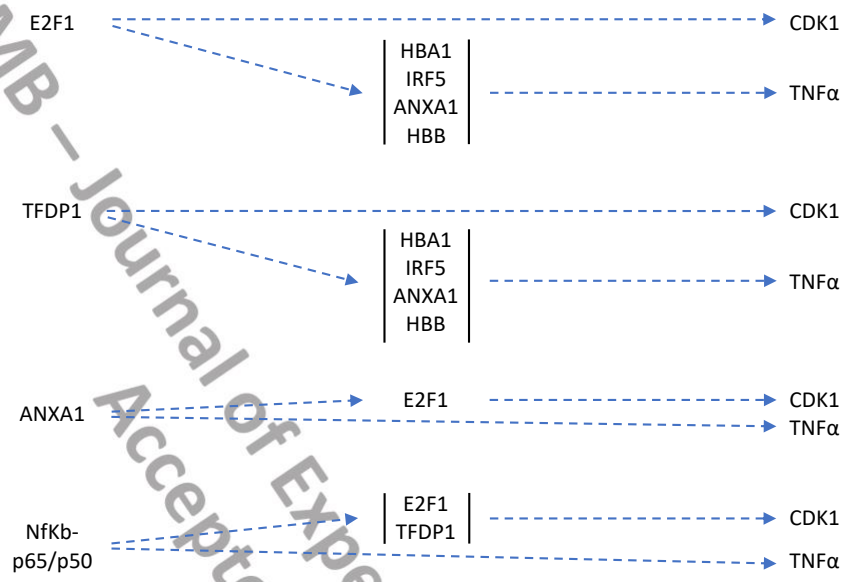

Figure 7. Evaluation of signaling pathways linkage between *cdk1* and *tnf α* resulting in upregulation of both genes (⇢ indirect upregulation).

As result, we identified four interrelating regulators of *cdk1* and *tnf α* (E2F1, TFDP1, ANXA1 and NfKb-p65/p50) (Figure 7), four interrelating regulators of *cdk1* and *tp53* (E2F1, TFDP1, KLF4 and CBFbeta-MYH11) (Figure 8) and five interrelating regulators of *tnf α* and *tp53* (KLF4, CBFbeta-MYH11, ANXA1, IL1A and NfKb-p65/p50) (Figure 9).

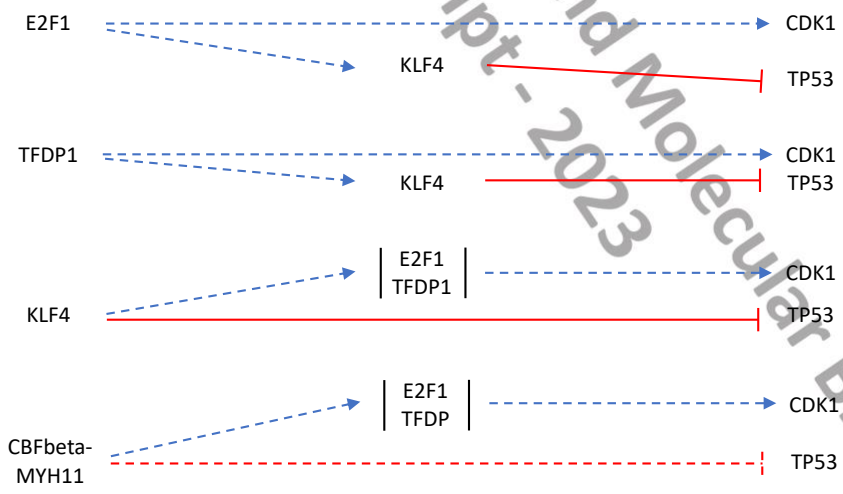

Figure 8. Evaluation of signaling pathways linkage between *cdk1* and *tp53* resulting in upregulation of *cdk1* gene and downregulation of *tp53* gene (⇢ indirect upregulation, ⊣ direct downregulation, ⇢⊣ indirect downregulation).

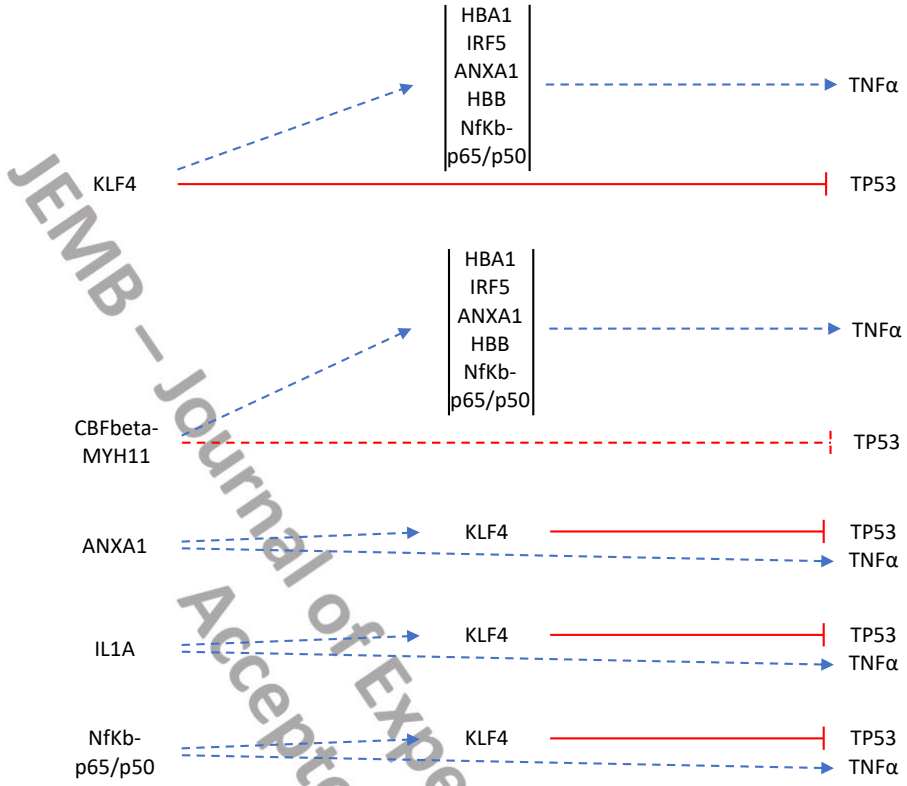

Figure 9. Evaluation of signaling pathways linkage between *tp53* and *tnf α* resulting in upregulation of *tnf α* gene and downregulation of *tp53* gene (⇢ indirect upregulation, ⊣ direct downregulation, ⇢⊣ indirect downregulation).

Analyzing the interrelating regulators of all 3 genes, we observed a common signaling pathway composed of TFDP1, E2F1 and KLF4, whose activity can simultaneously upregulate *cdk1* and *tnf α*, and downregulate *tp53* (Figure 10).

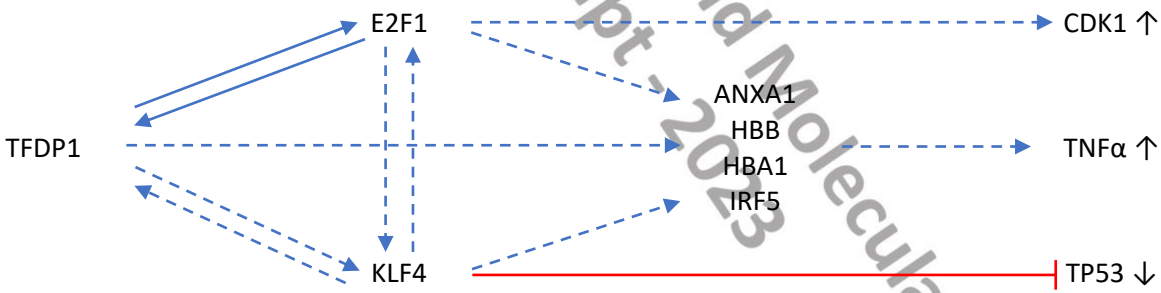

Figure 10. Evaluation of common signaling pathways linkage between *cdk1*, *tnf α* and *tp53* resulting in upregulation of *cdk1* gene, upregulation of *tnfα* and downregulation of *tp53* gene (→ direct upregulation, ⇢ indirect upregulation, ⊣ direct downregulation).

Discussions

Different methods of producing and administering CAP to tumor cells, yielding diverse and variable outcomes in terms of cytotoxic effects, proliferation rates, and modulation of various cellular signaling pathways have been evaluated in biomedical researches. These studies have also highlighted differences in treatment methods used and the effectiveness of CAP treatment (Volotskova et al. 2012; Keidar et al. 2013; Siu et al. 2015; Malyavko et al. 2020; Min et al. 2022). Although progress has been made in recent years regarding the mechanisms underlying the anti-tumor effects of CAP, they are still not fully understood, and further studies are needed to identify which cellular signaling pathways are modulated by CAP treatment (Turrini et al. 2017; Semmler et al. 2020; Tan et al. 2020; Motaln et al. 2021; Murillo et al. 2023). Therefore, in this study, we aimed to identify the cytotoxic effects of direct and indirect treatment with CAP generated from our custom-built source on human osteosarcoma cells. In addition, our objective was to evaluate the modifications that the treatment induces on the expression level of three representative genes involved in regulating the cell cycle and cell death. The results obtained in this study revealed that each experimental variant of the direct treatment with CAP had a drastic effect on reducing the viability of osteosarcoma cells, both at 2 hours and 24 hours after exposure, while the indirect treatment induced minor changes. In both treatment methods, the exposure time of 120s showed the most pronounced cytotoxic effect. Similar results of cytotoxicity from direct treatment with CAP have been observed in previous studies on various bone cancer cell lines (Gümbel et al. 2016; Gümbel, Gelbrich, et al. 2017; Saadati et al. 2018; Malyavko et al. 2020). However, regarding indirect treatment, unlike the results obtained in this study, previous studies show effects of reduced viability of bone tumor cells through the use of much longer treatment times (Canal et al. 2017; Saadati et al. 2018; Tornin et al. 2019; Mateu-Sanz et al. 2020; Xu et al. 2020; Hamouda et al. 2021). Interestingly, under certain treatment conditions, Biscop et al. obtained better cytotoxic results with indirect treatment compared to direct treatment (Biscop et al. 2019). Due to its superior efficiency demonstrated in this study, direct method was selected for CAP-treating HOS cells in order to evaluate changes in the expression levels of genes of interest. *cdk1* is a member of the cyclin-dependent kinase family and plays a crucial role in promoting the progression of the cell cycle through the G2/M phase in eukaryotic cells (Liao et al. 2017; Lemonnier et al. 2020). Our study results revealed that at 2 hours after direct CAP treatment, HOS cells exhibited a downregulation of *cdk1* gene which was not found to be correlated with the exposure time. Thus, we assume that HOS cells may undergo G2/M arrest due to the *cdk1* downregulation by CAP treatment (Badie et al. 2000; Yang et al. 2018). Surprisingly, at 24 hours post-treatment, upregulation of the *cdk1* gene was identified, this time in a dose-dependent manner, thus promoting the proliferation of the cell population that survived the CAP treatment (Zhang et al. 2018; Liu et al. 2022). Similar to our results obtained 2 hours post-exposure, Yan et al. detected a downregulation of *cdk1* gene in HepG2 cells 48 hours after direct treatment with CAP (Yan et al. 2010). While Nakai et al. showed increased levels of phosphorylated CDK1 protein and Lin et al. observed an elevated level of phosphorylated CDK1 protein and no effect on CDK1 protein levels after CAP treatment (indicating cell cycle arrest in G2/M phase due to the regulation of cyclin B1/CDK1 complex), Han et al. reported increased levels of CDK1 proteins in Human uterosacral ligament fibroblasts treated with CAP (Nakai et al. 2014; Lin et al. 2018; Han et al. 2021). To the best of our knowledge there are no studies regarding the evaluation of *cdk1* gene expression in CAP-treated osteosarcoma cells, apart from assessments considering the protein level reduction as a result of overexpression of the *p53, p21* and *bax* genes (Gümbel, Bekeschus, et al. 2017). Tumor necrosis factor alpha (TNF α) is a cytokine that exerts pleiotropic effects on various cell types. It is initially synthesized as a precursor form called transmembrane TNF α, which can function as both a ligand and a receptor. TNF α plays a role in the development of inflammatory and

autoimmune diseases and is involved in regulating cytotoxic activity against normal and tumoral cells, being part of the extrinsic apoptosis pathway (Cavalcante et al. 2019a; Mercogliano et al. 2020; Jang et al. 2021). Although according to the Dunnett post hoc test, the differences observed in the levels of *tnf α* gene expression within the three experimental variants compared to the control sample were not statistically significant, following CAP treatment, a modulation of *tnf α* gene expression similar to that of *cdk1* was observed. Specifically, downregulation of *tnf α* was observed at 24 hours post-treatment, followed by its upregulation at 24 hours post-treatment. Previous studies have reported overexpression of *tnf α* following CAP treatment on tumor cells (Hou et al. 2015; Xia et al. 2019; Murthy et al. 2022), while studies regarding the ability of CAP to heal wounds have shown both increases and decreases in the expression level of the *tnf α* gene (Arndt et al. 2018; He et al. 2020). Due to the fact that TNF α has an ambivalent effect, either promoting cell death or cell survival (Wang et al. 2005; Kim et al. 2018; Kokolakis et al. 2021) it cannot be determined exactly whether the decrease in viability of HOS cells in this study can be correlated with the identified *tnf α* gene expression modulation. The tumor suppressor gene, *tp53*, plays crucial roles in various cellular processes such as apoptosis, cell cycle control, genomic stability, regulation of cell proliferation, metabolism, and immune response. *tp53* gene modulate the expression of more than 900 genes and has the ability to induce apoptosis through both intrinsic and extrinsic pathways (Gümbel, Bekeschus, et al. 2017; Cavalcante et al. 2019b; Marvalim et al. 2023; Wang et al. 2023). The CAP treatment of HOS cells induced downregulation of the *tp53* gene at both 2 and 24 hours after treatment, except for HOS cells exposed to CAP for 30 seconds and evaluated at 2 hours post-treatment, where upregulation of *tp53* gene was recorded. Similar results were obtained by Shi et al., Eggers et al., and Adil et al., who identified low levels of *tp53* mRNA in various types of CAP-treated cells (Shi et al. 2017; Adil et al. 2020; Eggers et al. 2021). In contrast, other studies have reported increases in the *tp53* gene expression level in various tumor cells types as a result of CAP treatment (Turrini et al. 2017; Yadav et al. 2020; Sadoughi et al. 2021). According to the obtained results, we can state that treatment with CAP induces a decrease in the viability of HOS cells, but the mechanism of action appears to be independent of *tp53*. By comparatively evaluating the results obtained from assessing the expression of *cdk1*, *tnf α*, and *tp53* genes in CAP-treated HOS cells, it can be presumed that there might be a connection between them determined by a direct or indirect regulatory mechanism of expression among these three genes or a common regulator whose activity modulates their level of expression. Additionally, we must also consider the possibility that, following CAP treatment, the expressions of the three genes are individually modulated through distinct signaling pathways. Analyzing the signaling pathways, we have observed that there is no direct or indirect connection between the three genes, meaning that the overexpression or underexpression of one of them does not influence the expression levels of the others. However, we have identified signaling pathways that include interrelating regulators, whose activation can lead to the regulation of *cdk1*, *tnf α*, and *tp53* genes expression. Therefore, we can assume that the effects of CAP treatment on the expression of *cdk1*, *tnf α*, and *tp53* genes in HOS cells could be based on the activation of one, two or all of the identified interrelating regulators. Further studies are necessary to evaluate the level of the proteins encoded by the analysed genes, and to confirm the activation of these interrelating regulators by CAP treatment. Moreover, new signaling pathways which promotes the proliferation or death of the cells should be evaluated in order to elucidate the underlying mechanism of CAP action on HOS cells.

Conclusions

CAP generated by our custom-build source greatly reduces HOS cells viability via direct treatment, whereas the indirect CAP treatment with PAM-PBS has negligible effects. In addition to its cytotoxic effects, direct treatment modulates *cdk1* gene expression, leading to cell cycle arrest of HOS cells at two hours after the treatment. However, an opposite effect is observed at twenty-four hours following treatment. According to the Dunnett post-hoc test the expression level of the *tnf α* showed no statistical significance. Our results revealed that the molecular mechanism of cells death in HOS treated cells does not involve the expression of *tp53* gene, on the contrary, direct CAP treatment downregulate *tp53* gene expression. Furthermore, we emphasize the need to further evaluate caspases signaling pathways to establish the molecular mechanism modulated by direct CAP treatment, produced with our custom-build plasma source, which induces cell death in HOS cells. The analysis of interrelating regulators for the 3 interest genes showed that the *cdk1* and *tnf α* can be simultaneously upregulated, while *tp53* will be downregulated.

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Supplementary files

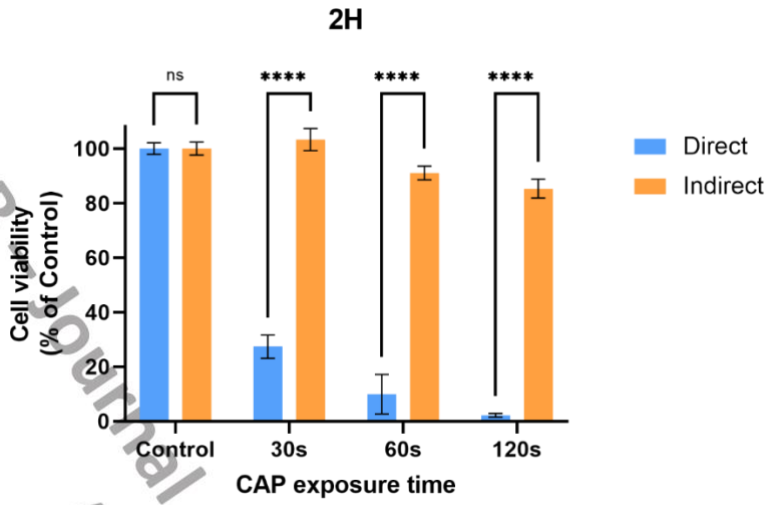

Figure S1. Comparison between the viability of HOS cells directly and indirectly treated with CAP, 2 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by two-way ANOVA followed by Šídák's post hoc multiple comparison test (ns – nonsignificant, **** p<0.0001).

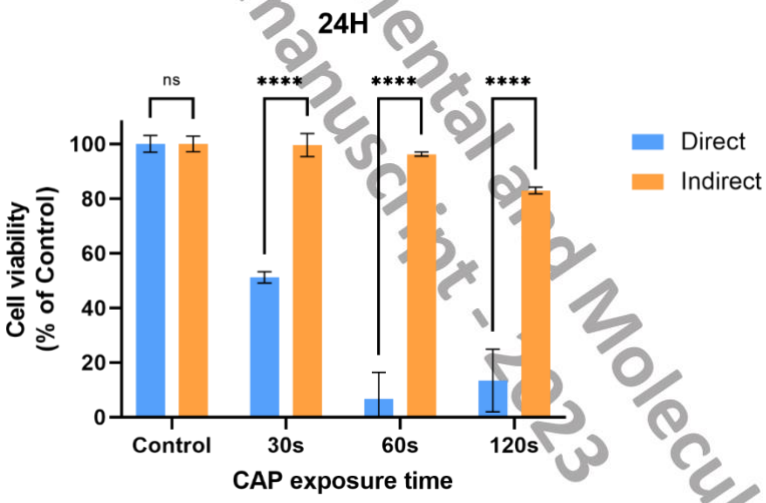

Figure S2. Comparison between the viability of HOS cells directly and indirectly treated with CAP, 24 hours post-treatment. Values are means ±SEM (n=3). Statistical significance was determined by two-way ANOVA followed by Šídák's post hoc multiple comparison test (ns – nonsignificant, **** p<0.0001).