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Time-dependent modulation of MC3T3-E1 pre-osteoblasts by a helium cold plasma: a preliminary study on its potential relevance for alveolar bone regeneration

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Keywords: cold atmospheric plasma, periodontitis, osteoblasts, bone tissue regeneration

Supplementary material for this article is available online

Abstract

Cold atmospheric plasma (CAP) has demonstrated promising antimicrobial activity and potential to promote tissue regeneration. However, most existing research has focused on its effects on soft tissue cells, such as fibroblasts, while its influence on osteoblasts, the main cells responsible for alveolar bone formation, is rather limited. As a first step towards addressing this gap, the present study assessed the biological effects of a newly custom-designed CAP source on the pre-osteoblastic MC3T3-E1 cell line, with a primary focus on the cellular survival and osteogenic differentiation. Through the use of diverse *in vitro* methods, the cytotoxicity (e.g. Live&Dead and CCK-8 assays; morphological characterisation) and osteogenic differentiation potential (e.g. alkaline phosphates activity and Alizarin Red S staining) of a helium-based CAP were assessed. The cytotoxicity results indicated a treatment duration- and exposure number-dependent response, i.e. repeated CAP exposures within the 30 s–60 s range yielded the highest cell survival and metabolic activity, while extended exposures (180 s–240 s) significantly reduced cell viability by approx. 30%. On the contrary, an opposite trend was observed in terms of osteogenic differentiation, where repeated, prolonged exposures (90 s−120 s), resulted in an enhanced matrix mineralisation, suggesting an increased level of late-stage osteogenic differentiation. Overall, these findings indicate that CAP treatment can modulate the MC3T3-E1 pre-osteoblasts' behaviour in a dose-dependent manner, suggesting a potential relevance for bone regeneration applications, and possibly for alveolar bone regeneration. Importantly, this study represents an initial effort towards the exploration of the effects of CAP exposure on osteoblasts using a pre-osteoblastic cell line and highlights the need for further research through more in-depth studies (e.g. periodontal-specific cell models and *in vivo* studies) prior to clinical translation.

1. Introduction

Periodontitis is a multifactorial, polymicrobial inflammatory disease of the oral cavity characterised by the destruction of the tooth-supporting tissues, i.e. the periodontal ligament and alveolar bone [1]. The pathogenesis of the condition is very complex, and involves a dynamic interplay between specific microbial pathogens, a destructive host immune response, and, according to a large body of literature, the existence of both congenital (sex, genetic predisposition, teeth anatomy) and acquired (smoking, pre-existing systemic conditions, obesity, stress, nutrition, etc) risk factors [1]. Thus, in a susceptible host, lipopolysaccharide and other virulence factors secreted by key periodontal pathogens such as *Porphyromonas gingivalis, Fusobacterium nucleatum, Aggregatibacter actinomycetemcomitans, Tannerella*

forsythia and *Treponema denticola* [2] trigger an overactive immune response. This leads to the secretion of a wide selection of pro-inflammatory cytokines such as interleukin (IL)-1β, tumour necrosis factor-α and prostaglandin E_2 (PGE_2) [3]. Together with the virulence factors, these cytokines stimulate matrix metalloproteinases production by the oral cavity-derived cells, resulting in the destruction of the periodontal collagen [4]. Moreover, during inflammation, pro-inflammatory cytokines stimulate the expression of receptor activator of nuclear factor kB ligand (RANK-L) on osteoblasts, osteocytes, and other cell types, including the activated T helper cells, stromal cells and the periodontal ligament fibroblasts [5, 6]. Once expressed, RANKL interacts with the receptor activator of nuclear factor kB (RANK) located on the surface of osteoclast precursors, promoting their differentiation and maturation. In the end, the mature, activated osteoclasts will mediate the destruction of the alveolar bone [7]. Clinically, these cellular and molecular changes translate into a damaged periodontium [8] that left untreated or inadequately managed, can lead to tooth loss, a decreased quality of life and an increase in the overall healthcare costs [9].

Depending on the disease's severity, the conventional treatment plan for periodontal patients comprises of a variation of non-surgical and surgical approaches [10]. Subgingival scaling and root planing are primary non-surgical procedures which aim to reduce the microbial load and inflammation, thus halting the progression of the condition [11]. However, the complete calculus removal is often unattainable, especially in anatomically complex regions [11]. Moreover, emerging evidence suggests that bacterial mediation of neutrophil extracellular traps may contribute to calculus resistance to enzymatic degradation, promoting its persistence within the oral cavity [12, 13]. This mechanism may partly explain the high recurrence of periodontopathogen recolonisation following treatment, thus limiting the long-term efficacy of these approaches [14]. To address this issue, adjunctive therapies such as topical or systemic antibiotics and chlorhexidine-based mouthwashes are commonly prescribed [15], but their clinical use is often limited by antimicrobial resistance, insufficient tissue penetration and systemic side effects [16]. In situations when the deep periodontal pockets persist, surgical interventions that provide an improved access for debridement are recommended, but similar to any surgical procedure, are associated with postoperative complications [17].

In addition to reducing the microbial load and modulating the host's destructive immune response, an effective management of the condition also includes the complete regeneration of the periodontium through procedures such as guided tissue regeneration and bone grafting [18], which aim to preserve/restore the damaged tissues. However, despite the extensive evidence supporting the clinical benefits of these procedures, the clinical outcome remains variable and can be influenced by various patient- and defect-related factors [18]. Thus, while conventional non-surgical and surgical therapies often yield clinical improvements, the healing response is typically limited and quite unpredictable. Consequently, the investigation of alternative approaches capable of overcoming current therapeutic shortcomings represent a key area of ongoing research.

In this context, CAP has emerged as a promising treatment modality, offering unique biological effects that may support both antimicrobial action and tissue regeneration. CAP is a weakly ionised gas generated by electric discharge in the form of a plasma jet containing ground-state and excited atoms, free radicals and molecules, charged particles, ultraviolet (UV) light and reactive species [19]. A key advantage of CAP lies in its generation under ambient conditions and at atmospheric pressure, which ensures that its overall temperature remains close to physiological levels, allowing for its safe application to biological tissues without causing thermal damage, thereby supporting its use in various biomedical contexts [20]. Moreover, through the production of reactive oxygen and nitrogen species (RONS), CAP can exert several biological effects, such as antimicrobial activity against various pathogenic agents [21–24], promotion of tissue regeneration [25–29], immune response modulation [15, 25, 28], and programmed cell-apoptosis and cell arrest in various cancer models [30–34]. Based on this, CAP may represent a promising technique that integrates the therapeutic properties required for an efficient management of periodontitis.

Building upon the positive results observed in a previous study on gingival fibroblasts and macrophages [25], we decided to further purse this research direction and focus on a different, key cell type found in the periodontium, namely osteoblasts. For this purpose, commercially available MC3T3-E1 pre-osteoblast have been used as an *in vitro* model to o investigate the effect of the newly developed helium-based CAP on cellular viability and osteogenic differentiation under different experimental conditions. Although this *in vitro* model does not encompass the full complexity of the periodontal environment, it provides a controlled framework to explore the direct pro-osteogenic effects of CAP, which may have a potential relevance for bone healing and, possibly for alveolar bone regeneration.

While previous studies conducted by other research groups [26, 35, 36] have examined the regulatory effects of CAP on osteoblast-like MC3T3-E1 cells, the reported results are variable and the experimental approaches are highly heterogeneous. This

phenomenon can be attributed to the fact that the observed biological responses are strongly dependent on plasma source configuration and treatment parameters. Therefore, differences in gas composition, discharge type or exposure conditions can lead to distinct cellular outcomes, even when the same *in vitro* model is used. Moreover, the majority of the previously reported studies have focused on single, short-duration CAP exposures, which may not fully reflect the clinically relevant conditions. Since prolonged cellular activation is often requited for regenerative applications, the cumulative effects of repeated CAP treatments over extended periods should also be investigated. In addition, few studies have explored the use of helium as the noble gas in CAP, despite its distinct advantages over other gases in terms of non-thermal effects and tissue compatibility [37–40].

Therefore, the aim of the present study was to investigate the effects of a custom-designed helium-based CAP source on the viability and osteogenic differentiation of the MC3T3-E1 pre-osteoblasts under different experimental conditions. In particular, the study investigated whether repeated CAP exposure provides greater biological benefits than the single, short-duration application. To address this question, the cells were exposed to different CAP treatment regimens and their biological response was evaluated in terms of cellular survival, morphological characteristics, as well as of osteogenic differentiation potential. By addressing the current gap that exists in literature, this study provides a preliminary insight into the potential of repeated helium-based CAP treatment to modulate osteoblast behaviour in a manner that is relevant for bone regeneration applications and, possibly, for future periodontal regenerative therapies.

2. Materials and methods

2.1. CAP operational setup

The CAP treatment was conducted using the PlasmaBIC device, a compact, hand-held, homemade double-dielectric plasma jet source designed and produced in the laboratories of the University of Milan-Bicocca (Milan, Italy), as described in our previous work [25]. The system consisted of a central copper electrode enclosed in a glass capillary (external diameter 1.4 mm) that was housed within a gas nozzle (internal diameter 6 mm), with an external grounded ring which completed the configuration. The electrode was connected to a transformer (1:100 ratio) driven by a 100 kHz square-wave signal with a 50% duty cycle, and a 1 kHz modulation at 20% duty cycle in order to prevent overheating. In operating conditions, the flow rate of the carrier gas helium was adjusted to 2 slpm and the set voltage was that of 2 kVpeak.

As previously reported [25], the power transferred to the plasma has been measured to be less than 181 ± 1 mW. In the same work the vibrational and rotational temperatures of nitrogen have been evaluated from Optical Emission Spectroscopy measurements: while the vibrational temperature has been measured to be high (3500 ± 100 K), the rotational temperature has been observed to remain close to room temperature (390 ± 30 K).

In a set of preliminary studies, various distances from the bottom of the well to the tip of the plasma source nozzle were examined and in the end a distance of 1.5 cm was chosen for future experiments. During the preliminary characterisation, the stationarity of the plasma source operational parameters has been checked. Specifically, the gas flow, being set by a gas flow controller, was constant by design, whereas the voltage constancy has been explicitly checked to rule out possible drifts in the power supply electronics. The outcome was that no variations in the power supply behaviour were observed even in long operations.

2.2. Cell culture and treatment plan

Commercially available MC3T3-E1 murine pre-osteoblasts purchased from American Type Culture Collection (ATCC, Manassa, VA, USA) were grown in Dulbecco's Minimal Essential Medium (DMEM, Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with foetal bovine serum (10%, FBS, Life Technologies Corporation, Grand Island, NY, USA) and 1% (v/v) penicillin (10000 units ml^{-1})/streptomycin (10 mg ml^{-1}) (Sigma-Aldrich Co., St. Louis, MO, USA), and maintained in a humidified atmosphere with 5% CO_2 at 37°C. At a confluence of around 80%, the osteoblasts were seeded in technical triplicates in 96-well tissue culture plates (TCPS) with a growth surface of 0.342 cm^2/well, at varying initial densities, based on the preliminary experiments and subsequent biological assessment. Thus, in a first instance for the viability and morphological studies, the cells were seeded at an initial density of 1×10^4 cells cm^{-2}, while for the osteogenic differentiation, the used cell density was of 4×10^4 cells cm^{-2}. It is important to mention that the differentiation studies were performed under osteogenic conditions, in which the standard culture medium was supplemented with 50 μg ml^{-1} ascorbic acid (Sigma-Aldrich Co., St. Louis, MO, USA), 5 mm β-glycerophosphate (Sigma-Aldrich Co. St. Louis, MO, USA) and 10^{-8} M dexamethasone (Sigma-Aldrich Co. St. Louis, MO, USA). In addition, to prevent cellular stress and death, the osteogenic media was changed every two to three days over the course of the experiment.

The CAP treatment was conducted after 24 h in culture, time period necessary for the cells to adhere to the bottom of the well. Afterwards, two

Table 1. Sample notation.

Sample	Notation
CAP-single treatment	CAP Tr I
CAP-multiple (3*x*) treatment	CAP Tr III
Control group without CAP treatment	TCPS
Control group without CAP treatment in standard culture medium	TCPS (-)
Control group without CAP treatment in pro-osteogenic medium	TCPS (+)

experimental treatment conditions were established, namely direct exposure for one day and 3 d consecutively to CAP following the previously established time points of 30 s, 60 s, 90 s, 120 s, 180 s and 240 s. It is important to mention that for all of the conducted experiments, CAP was applied directly to the culture medium covering the cells (100 μl/well), with the medium being changed daily prior to CAP exposure. In the repeated treatment groups, CAP was applied once per day. Moreover, for the osteogenic differentiation experiments, the cells have been kept under osteogenic stimulation during plasma exposure.

Given the volume of medium covering the cells (100 μl) and the direct application of CAP, potential thermal and evaporation effects were considered. Although these parameters were not explicitly monitored, our previous study [25] showed that the plasma source used in this study does not induce measurable temperature changes of the substrate during the treatment. Accordingly, no attempt was made to first treat the medium and subsequently add it to the cells. This is because this low-power source is not optimal for such an indirect treatment, where the requirement of negligible heating is not so stringent. Furthermore, for the envisaged application, a direct plasma treatment appears much more suitable. Table 1 indicates the notations of the sample used throughout the study.

2.3. Pre-osteoblasts survival and metabolic activity assessment

To evaluate the viability of MC3T3-E1 cells following CAP treatment, a commercially available LIVE/DEAD viability/cytotoxicity kit (Molecular Probes, L-3224, Eugene, OR, USA) was utilised according to the protocol described in [41]. After staining, the fluorescent labelled cellular monolayer was examined using an inverted fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan), and representative images were captured using the cellSense dimension imaging software (version 4.1). To complement and confirm these qualitative observations, a quantitative assessment of metabolically active viable cells was conducted using the cell counting Kit-8 (CCK-8) assay (Sigma-Aldrich Co., St. Louis, MO, USA), following the method previously reported in [42].

2.4. Pre-osteoblasts morphology image analysis

To determine whether the CAP treatment affects the MC3T3-E1 cells' morphologies, the fluorescent staining of the actin cytoskeleton with AlexaFlour 488-conjugated phalloidin (20 μg ml-1, Invitrogen, Eugen, OR, USA) was also performed, as previously reported [43]. Similar to the viability assessment, the Olympus IX71 (Olympus, Tokyo, Japan) inverted microscope equipped with fluorescence and imaging software cellSense dimension (version 4.1) were used to visualise the labelled cells and capture the representative fields, respectively. Furthermore, in order to offer a quantitative interpretation, the spreading area of the MC3T3-E1 cells exposed to CAP was analysed using the Image J software (version 1.53 c, National Institutes of Health, Bethesda, MD, USA). For this, ten random fields were acquired per experimental group, and all cells within each microscopic field were measured individually by tracking the contour of each cell manually with the help of the freehand selection tool. For statistical analysis, the mean cell area was calculated for each field, and these field-level means ($n = 10$ fields per group) were used for comparisons between groups. To minimise operational bias, identical tracing criteria have been applied for all of the measurements: the analysis was performed by a single person; only fully visible cells were included; overlapping or partially visible cells were excluded; ImageJ display settings and zoom levels were consistent throughout the analysis.

2.5. Pre-osteoblast differentiation investigation

In the present study, the effects of CAP treatment on the osteogenic differentiation of the MC3T3-E1 cells was assessed by analysing the two main determinants of pre-osteoblast differentiation into mature osteoblasts, namely alkaline phosphatase (ALP) activity and extracellular matrix mineralisation.

2.5.1. Intracellular ALP activity determination

The ALP activity was determined in cell lysates at 10 d and 20 d after final CAP treatment. For this purpose, the ALP Activity Colorimetric Assay Kit (BioVision, Milpitas, CA, USA) was used in accordance with the manufacturer's instructions [44]. Briefly, to initiate the reaction, 80 μl of cell lysate containing the equal amounts of proteins was used, to which 50 μl of 5 mM pNPP solution was added. The reaction mixture was then incubated at room temperature for 60 min in the dark. At the end of the incubation period, the reaction was stopped by adding 20 μl of STOP solution, and the optical density (OD) of the resulting product was measured at 405 nm using an automated plate reader (FlexStation 3 multi-mode microplate reader, molecular devices, San Jose, CA, USA).

To determine the ALP phosphatase activity, the obtained values were compared to a standard curve and in order to avoid variations due to different protein concentrations, all samples were normalised to

the total protein content per well, which was previously determined using the Bradford assay.

The final ALP activity was calculated as the amount of pNP produced per μg protein per minute using the following formula:

$$\text{ALP activity}(\text{U}/\mu\text{gprotein}/\text{min}) = \text{A}/\text{protein}/\text{T},$$

where A represents the amount of pNP produced by the samples (in μmol); protein is μg protein and T is the reaction time in minutes (60 min).

2.5.2. Extracellular matrix mineralisation assessment

The mineralisation of the extracellular matrix was investigated at 28 d and 42 d after final CAP treatment by staining the calcium deposits with an Alizarin Red S solution (Sigma-Aldrich Co., St. Louis, MO, USA). Briefly, the cellular monolayer was washed twice with a phosphate buffer saline (PBS) solution, fixed with a solution of 10% paraformaldehyde (PFA) for 20 min (room temperature), washed thoroughly with distilled water and incubated for 60 min, at room temperature, with 100 μl/well Alizarin Red S solution. Afterwards, the dye was removed, and the stained mineralisation nodules were observed under an inverted microscope (Olympus IX71, Olympus, Tokyo, Japan), while the representative fields were captured with the help of the imaging software cellSense dimension (version 4.1). To quantify the degree of mineralisation, the wells were air dried and 100 μl of 5% perchloric acid solution was added to dissolve the calcium deposits, while the absorbance was measured at 405 nm with an automatic plate reader (FlexStation 3 multi-mode microplate reader, Molecular devices, San Jose, CA, USA).

2.6. Statistical analysis

The statistical analysis of the obtained data was performed using the GraphPad software (version 8, GraphPad, San Diego, CA, USA) using either one-way ANOVA or two-way ANOVA with Bonferroni's multiple comparison post hoc test to determine differences among groups. All experiments were performed in technical triplicates ($n = 3$), with the results presented as mean $\pm$ standard deviation (SD) and differences considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Pre-osteoblasts survival and metabolic activity under CAP treatment

Given that in tissue regeneration-based therapies, maintaining the viability of the healthy cells is a fundamental factor through which the proper structure and function of the affected tissue is regained and that of the newly regenerated tissue is assured, the potential cytotoxic effects of CAP treatment applied directly onto the cultured pre-osteoblasts, in different experimental conditions, were assessed by combining the qualitative calcein acetoxymethyl ester (AM)/ethidium homodimer (EthD)-1 labelling with the quantitative CCK-8 assay. Figure 1 highlights the ability of CAP to sustain cell viability, by presenting a cellular monolayer dominated by viable green-stained cells, regardless of the experimental condition. It is important to mention that due to the low cytotoxicity of the CAP source the number of red-labelled dead cells was minimal, resulting in little to no observable red fluorescence in the representative fields. To confirm the microscopic observations, a CCK-8 assay was also conducted and the results are presented in figure 2. The graphical representation indicates a downward trend in the number of viable metabolically active cells directly proportional with the exposure time-periods (240 s $<$ 180 s $<$ 90 s $<$ 60 s $<$ 30 s), phenomenon observed more pronounced in the case of the multiple (3x) CAP applications (approx. 30%). This observation is also supported by the LIVE/DEAD test's results, where the cell treated for 180 s and 240 s displayed a decrease in cell density when compared to the control group and the other exposure periods. In terms, of comparing the efficiency of the single vs. multiple (3x) CAP treatments on the metabolic activity of the MC3T3-E1 cells, the CCK-8 assay revealed a 1.5-fold increase (approx. 50%) in the OD values recorded by the groups treated three days consecutively ($p < 0.0001$ vs CAP single treatment), regardless of the exposure periods. Overall, these findings demonstrate that even after multiple applications, the short CAP exposures are well tolerated by the MC3T3-E1 pre-osteoblasts. These findings align with previously published studies reporting on the non-cytotoxic effects of CAP on bone-derived cells. For instance, Nitsch *et al* [30] observed a slight decrease in the human osteoblasts' viability and proliferation rates with increasing treatment duration, whereas Tominami *et al* [26] reported no significant differences in the MC3T3-E1 cells proliferation rates for any of the tested plasma exposure conditions. Overall, these results indicate that the short-term plasma exposure is not expected to induce deleterious effects in osteoblastic cells, supporting its feasibility as a potential medical device with real clinical applications. However, the cellular response of the bone-derived cells can vary significantly depending on several factors, including the CAP device used, the origin of the bone cells (e.g. animal vs human), the experimental conditions (e.g. exposure times, frequency), and the treatment method (direct vs indirect application). Choi *et al* [35] further highlighted this variability, demonstrating that the method of CAP application can significantly affect MC3T3-E1 cells' viability and proliferation. Thus, at 24 h post-treatment, both the direct treatment (DT) and plasma activated media (PAM) groups showed increased proliferation

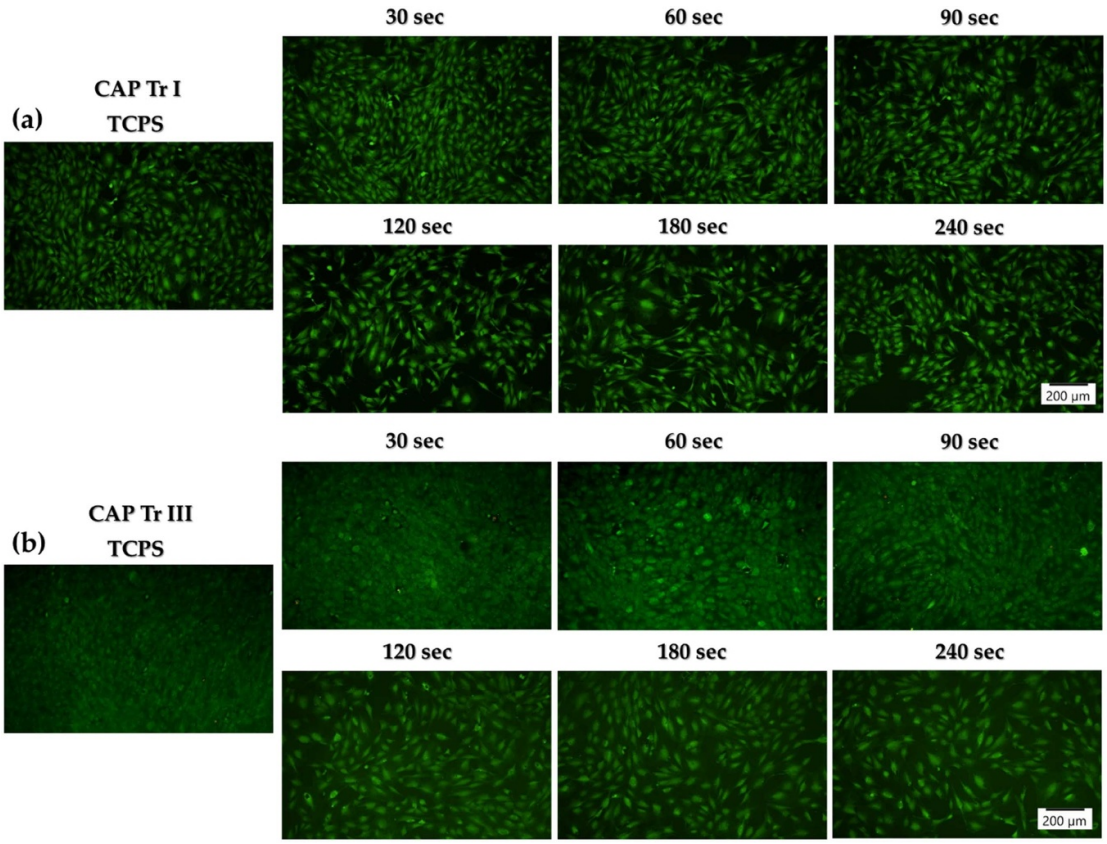

Figure 1. The survival rate of the MC3T3-E1 cells exposed to single (**a**)/multiple (**b**) CAP applications, as assessed by the LIVE/DEAD assay at 24 h post-treatment (live cells: green fluorescence; dead cells: red fluorescence). The size of the scale bar is 200 μm.

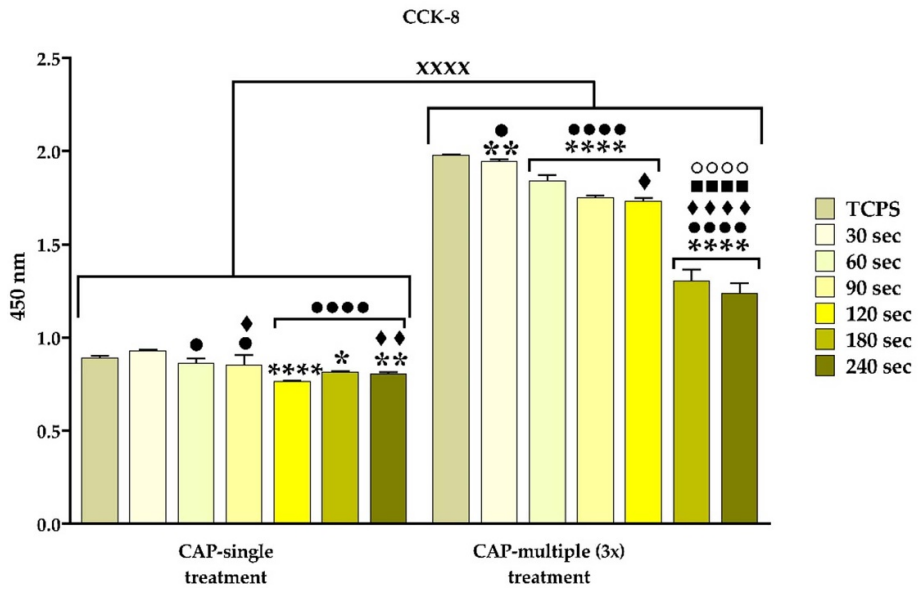

Figure 2. The cellular viability of the MC3T3-E1 cells measured as mitochondrial metabolic activity using the CCK-8 assay, following single/multiple CAP applications after 24 h post-treatment. The results are expressed as means ± SD ($n = 3$, **** $p < 0.0001$, ** $p < 0.01$ and * $p < 0.05$ vs TCPS; ●●●● $p < 0.0001$ and ● $p < 0.05$ vs 30 s; ♦♦♦♦ $p < 0.0001$, ♦♦ $p < 0.01$ and ♦ $p < 0.05$ vs 60 s; ■■■■ $p < 0.0001$ vs 90 s; ○○○○ $p < 0.0001$ vs 120 s). The significance level between the two groups: xxxx $p < 0.0001$ vs CAP single treatment.

compared with the non-treated control (NT) group, whereas after 48 h, the DT with media change (MC) and PAM groups were the ones showing a significant increase in their proliferation rate—approx. 20% higher than the NT group. Moreover, since the biological effects of CAP are primarily attributed to the

generation of reactive oxygen species (ROS) and since the physical parameters of CAP such as power, voltage, electromagnetic field, etc, vary greatly across different devices, the concentration and composition of generated ROS also differ [45]. Consequently, these variations in ROS profiles can lead to different biological responses in bone-derived cells, emphasizing the importance of standardizing CAP parameters when evaluating its biomedical applications.

3.2. Pre-osteoblasts morphological features

Since cell morphology is closely linked to cell function [46], in the next experimental step, the morphological adaptations of the MC3T3-E1 cells treated with CAP were microscopically investigated via the fluorescent labelling of the cytoskeleton actin fibres with AlexaFlour 488-conjugated phalloidin. As shown in the fluorescent microscopic images, the cells exposed to a single CAP application exhibited morphological characteristics comparable to those observed in the control group. Specifically, the cells presented a typical polygonal and slightly elongated body shape, with well-defined stress fibres primarily oriented parallel to the longitudinal axis of the cells and extending into cellular protrusions (figure 3(a)). At the same time, repeated CAP exposures did not induce notable morphological alterations in most treatment groups, which retained a similar morphological appearance as the control culture. However, an exception was observed in the case of the pre-osteoblasts subjected to multiple treatments at durations of 180 s and 240 s, which showed a significantly greater degree of cell spreading relative to both the control and other treatment groups (figure 3(b)). It is important to note that this enhanced spreading was accompanied by a noticeable reduction in cell density, which aligns with the viability results. This phenomenon suggests a decline in the cell proliferation rates, particularly under longer CAP exposure conditions (180 s and 240 s). Furthermore, the morphometric semi-quantitative analysis of cell area spreading (figure 4) corroborates the morphological observations. Thus, it was noted that the cells exposed to CAP for 180 s and 240 s presented larger spreading areas and more elongated bodies when compared to the control group and the cells treated for shorter time periods. Moreover, this effects became more pronounced following repeated CAP treatments. It is critical to recognised that the comparatively large error bars for some conditions reflect the heterogeneity of cell sizes and spreading areas within the cell culture. Thus, in order to represent the real biological variability of the culture, we measured all cells present in the microscopic field rather than selecting only a subset.

Based on literature evidence that CAP, through its ability to generate charged particles such as ions and electrons, can induce protein alterations and structural modifications in bacterial systems [47], we speculated that similar electrostatic effects may have a potential influence on various cellular components. While electrostatic interactions are essential for a wide range of cellular processes, including cytoskeleton organisation [48], an excessive electrostatic energy may not always be beneficial. In this context, we propose that the morphological modifications observed following the repeated CAP exposure may reflect an indirect cellular response to altered electrostatic conditions. However, no direct experimental evidence demonstrating that CAP can alter the cytoskeletal structure of pre-osteoblasts has been reported to date. Therefore, this interpretation remains however purely hypothetical, and further in-depth studies are necessary to validate whether CAP-induced electrostatic changes can indeed modulate the cytoskeleton organisation and other cellular processes such as adhesion, migration and proliferation.

On the other hand, it is important to mention that an indirectly proportional relationship between cell density and spreading area is a well-documented phenomenon, even in the absence of external stimuli [49]. As cells become more sparsely distributed, they naturally tend to spread more extensively due to the increased availability of space and reduced competition for substrate adhesion sites. This suggests that the enhanced spreading observed under these conditions may, at least in part, reflect the general density-dependent behaviour rather than being solely attributed to the direct effects of CAP.

3.3. Pre-osteoblasts differentiation

Given that osteogenic differentiation of bone cells is a crucial process for bone tissue regeneration, this study aimed to evaluate the effects of CAP on pre-osteoblast differentiation by assessing two bone-specific markers, namely ALP activity and calcium nodule formation, at 10 d and 20 d, and 28 d and 42 d, respectively, after the final CAP application. It is worth noting that the experimental time points were chosen based on the established *in vitro* temporal progression of the osteogenic differentiation process. Specifically, early osteogenic commitment and matrix maturation occurs within the first two weeks of differentiation, during which the ALP activity is normally upregulated, turning the enzyme into a representative marker of early stage osteogenesis [50]. In contrast, matrix mineralisation is a late stage event that requires an extended culture time during which calcium deposition occurs [51].

3.3.1. Intracellular ALP activity

ALP is a ubiquitous, membrane-bound, dimeric metalloenzyme, expressed during osteogenic differentiation and strongly associated with the matrix maturation phase [50]. Evidence from literature suggests that the enzyme exerts a dual mechanistic function, enhancing matrix mineralisation

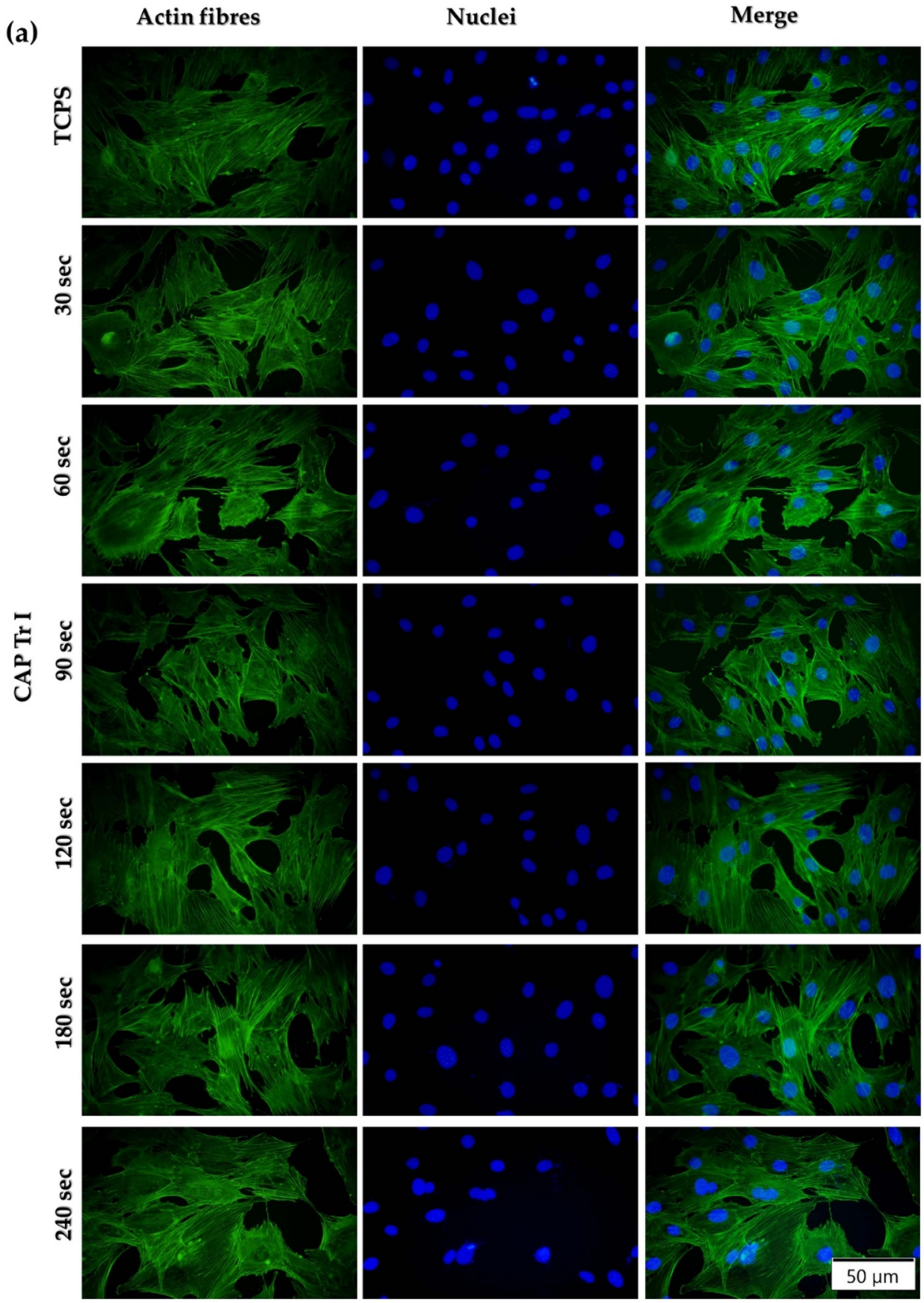

Figure 3. The morphological features of the MC3T3-E1 cells after single (**a**)/multiple (**b**) CAP applications, as assessed through the fluorescent labelling of the actin cytoskeletal protein (green fluorescence). DAPI was used to stain the nuclei (blue fluorescence). The size of the scale bar is 50 μm.

by increasing the local concentration of inorganic phosphate while concurrently decreasing the extracellular levels of pyrophosphate, a potent inhibitor of mineral deposition [52].

As seen from figure 5, at 10 d post-treatment, the MC3T3-E1 cells exposed to a single-CAP application exhibited an increased ALP activity compared to the control cells grown in the osteogenic medium without CAP treatment (TCPS (+)). However, this difference was not statistically significant. At 20 d post-CAP treatment, the differences in ALP activity are even more pronounced, both in comparison to the positive

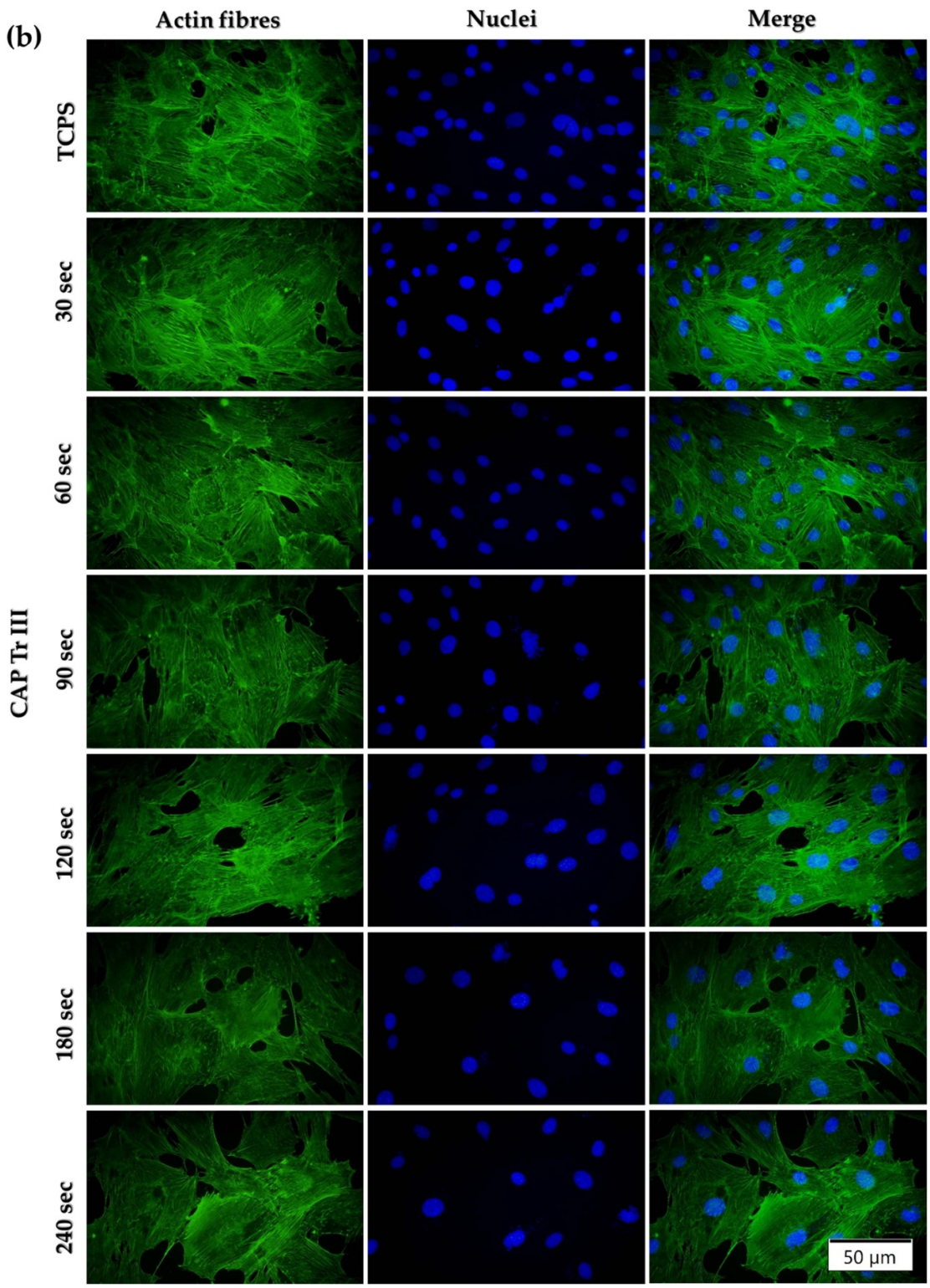

Figure 3. (Continued.)

osteogenic control group (TCPS (+)), and among the various CAP exposure durations. Notably, prolonged CAP exposures times of 90 s and 120 s led to a substantial increase in ALP activity compared to shorter application times (30 s and 60 s). In the case of the cell exposed to multiple (3*x*)-CAP applications, a similar upward trend in ALP activity was observed with increasing exposure time. However, this effect was noticeable only 20 d post-CAP treatment. Unlike the single CAP treatment, no significant differences were observed between the varying exposure durations when compared among each other ($p > 0.05$). Nevertheless, the ALP activity in this group still exceeded that of the positive osteogenic control ($p < 0.01$ and $p < 0.001$ vs TCPS *(+)*), with the exception of the group treated for 30 s, which

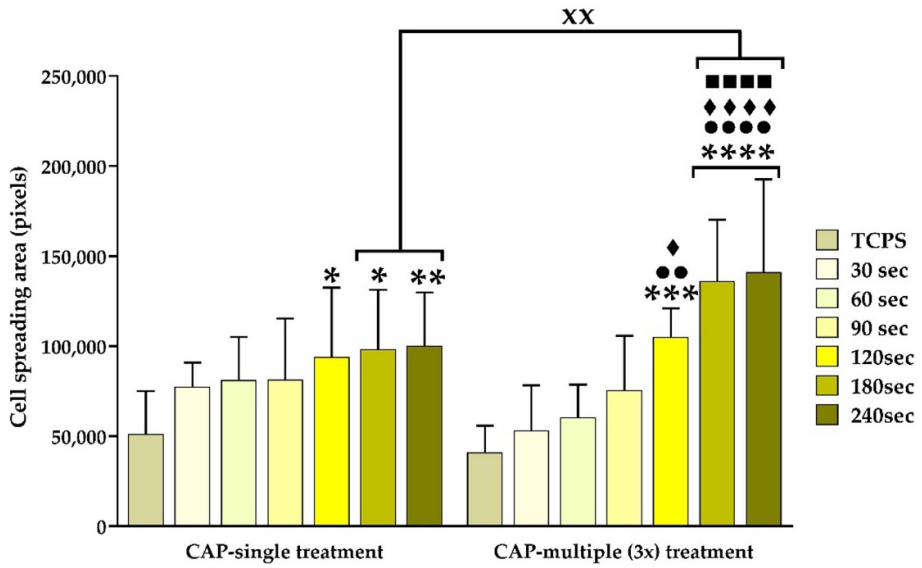

Figure 4. Quantification of cell spread area. Results are expressed as means ± SD ($n = 10$; **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ vs TCPS; ●●●● $p < 0.0001$ and ●● $p < 0.01$ vs 30 s; ♦♦♦♦ $p < 0.0001$, and ♦ $p < 0.05$ vs 60 s; ■■■■ $p < 0.0001$ vs 90 s;). The significance level between the two groups: xx $p < 0.0001$ vs CAP single treatment.

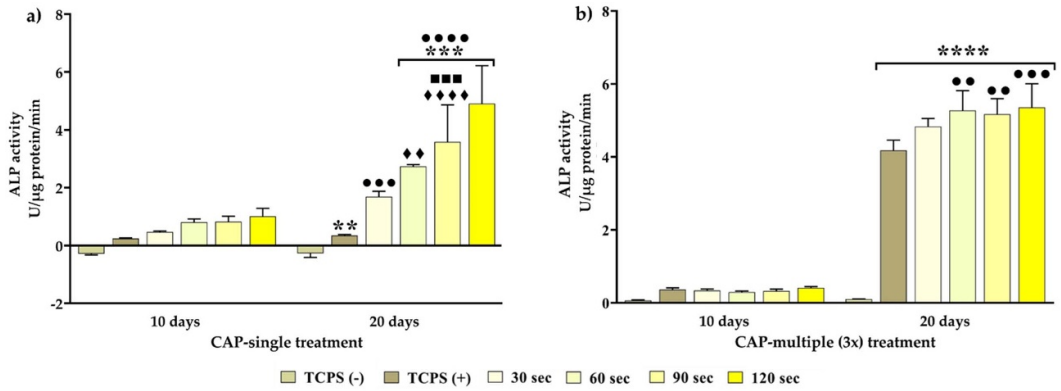

Figure 5. Intracellular ALP activity levels in MC3T3-E1 cells following single (**a**)/ multiple (**b**) CAP exposures, measured after 10 d and 20 d of incubation in pro-osteogenic differentiation medium. The control group TCPS (–) is excluded. ($n = 3$, mean ±SD, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ vs TCPS (-); ●●●● $p < 0.0001$, ●●● $p < 0.001$, ●● $p < 0.01$ vs TCPS (+); ♦♦♦♦ $p < 0.0001$ and ♦♦ $p < 0.01$ vs 30 s; ■■■ $p < 0.001$ vs 60 s).

showed a modest yet statistically significant reduction in ALP activity ($p < 0.01$ vs TCPS $(+)$). Moreover, the graphics reveal a marked increase in ALP activity between the post-treatment day 10 to day 20, in both experimental conditions. Overall, the results suggest the superiority of multiple CAP applications in promoting the differentiation of the MC3T3-E1 cells towards osteogenic cells (figure S1).

Similar to our findings, Tominami *et al* [26] demonstrated that plasma irradiation can increase ALP activity, and that its effectiveness is proportional to the number of irradiation repetitions. Thus, a higher number of plasma applications can be associated with a higher ALP activity, suggesting that, when applied appropriately, plasma treatment can promote MC3T3-E1 cell differentiation through ALP upregulation. In contrast, Egger *et al* [53] reported that a single CAP application had a beneficial effect on mineralisation by upregulating ALP activity in human calvaria osteoblasts, whereas triple application had a damaging effect and was therefore excluded from the study. A possible explanation for these contrasting results may be the different cell types used in the studies. For example, mouse-derived cells are known to be fast proliferating and exhibit a more rapid bone turnover compared to human cells [54]. Additionally, the differences in cellular behaviour may also arise from the distinct plasma sources and experimental conditions used. For instance, both of our study and that of Napp *et al* [24] used a helium-based plasma jet, while Eggers *et al* [53] manipulated a commercially available plasma device, i.e. ONE DENTAL/MEDICAL (Plasma MEDICAL SYSTEMS® GmbH, Nievern, Germany), which employs dielectric

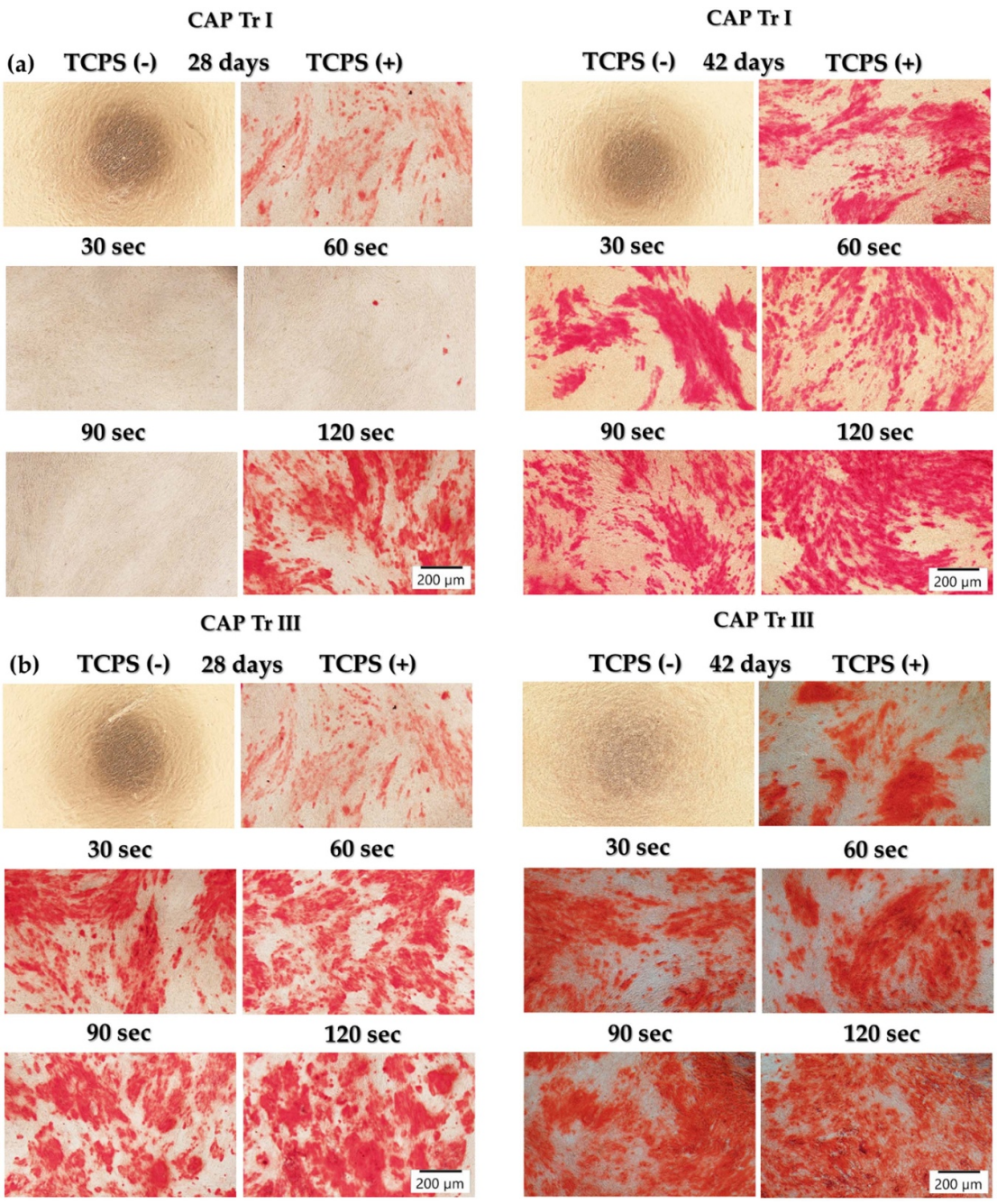

Figure 6. Optical microscopy images that highlight the mineralisation capacity of MC3T3-E1 cells exposed to a single (a)/ multiple (b) CAP applications after 28 d and 42 d of incubation in the pro-osteogenic differentiation medium (with the exception of the control group TCPS (−)), as evinced by the Alizarin Red S staining of the calcium nodules. Scale bar 200 μm.

barrier discharge technology to generate cold plasma from the ambient air. In this context, in order to fully elucidate the effects of CAP on osteoblast differentiation, further studies using the same plasma device on human-derived bone cells are necessary.

3.3.2. Mineralisation nodules formation

To further asses the ability of CAP to stimulate osteogenic differentiation, the formation of extracellular calcium deposits was also evaluated via Alizarin Red S staining at 28 d and 42 d post-CAP application. As new bone tissue begins to form, the activity of extracellular matrix proteins such as osteocalcin (OCN) increases, and mineral crystal, such as calcium, are deposited [55]. Based on this rationale, the presence of calcium nodules is commonly considered a hallmark of osteoblast maturation, and their *in vitro* detection serves as an indicator of late stage-osteoblast activity.

Figure 6 illustrates that cells subjected to multiple CAP applications exhibited a significantly greater accumulation of calcium deposits compared to those exposed to a single CAP application. In the single-treatment group, after 42 d in culture, the Alizarin Red S staining revealed the presence of calcium nodules in all of the experimental groups, with

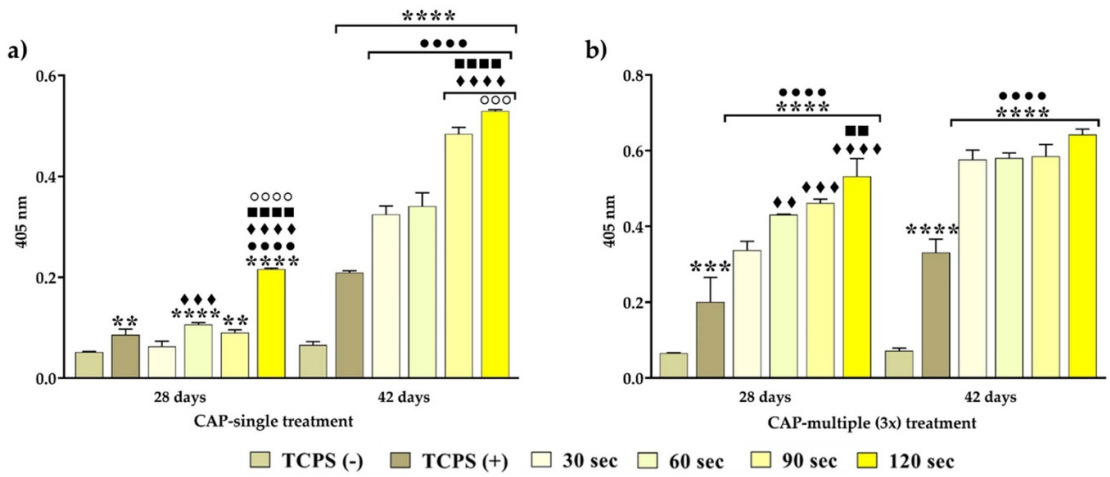

Figure 7. The effect of a single (**a**)/multiple (**b**) CAP applications on the osteogenic differentiation of MC3TE-E1 pre-osteoblast cells, as evaluated by spectrophotometric quantification of the solubilised calcium nodules (based on Alizarin Red S staining) ($n = 3$, mean ±SD, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ vs TCPS (-); ●●●● $p < 0.0001$ vs TCPS (+); ♦♦♦♦ $p < 0.0001$, ♦♦♦ $p < 0.001$ and ♦♦ $p < 0.01$ vs 30 s; ■■■■ $p < 0.0001$ and ■■ $p < 0.01$ vs 60 s; ○○○○ $p < 0.0001$ and ○○○ $p < 0.001$ vs 90 s).

the exception of the negative control (TCPS(−)). However, at 28 d post-treatment, mineralisation was observed exclusively in cells treated with CAP for 120 s. The microscopic results were consistent with the quantitative analysis of the solubilised calcium deposits, suggesting an improved osteogenic activity of MC3T3-E1 cells after multiple CAP applications (figure S2). Thus, similar to the ALP results, the calcium nodule deposition assay revealed significant differences between the analysed conditions, with the prolonged exposure durations of 90 s and 120 s exhibiting the highest OD values when compared to the control group ($p< 0.0001$ *vs TCPS (+)*) and the other CAP-treated groups (figure 7).

Even though the reported effects of ROS activation in osteogenic cells are often inconsistent and sometimes contradictory, the role of ROS in osteoblast differentiation is widely accepted. While excessive ROS levels can be harmful, moderate ROS levels are essential for various signalling pathways involved in cell proliferation, differentiation, and mineralisation [56]. For example, Han and Choi [57] reported that by treating the MC3T3-E1 cells with a non-thermal atmospheric pressure plasma, the generated RONS activated important signalling cascades involved in cell differentiation, such as mitogen-activated protein kinase (MAPK) pathway and the forkhead box O1 (FoxO1) transcription factor. Thus, the plasma-derived reactive species enhanced the phosphorylation of the p38 MAPK component, which in turn phosphorylated and activated FoxO1. This activation is particularly important, because p38-mediated FoxO1 signalling promotes the expression of essential osteogenic genes such as *Runx2*, ALP and OCN, thereby driving the progression of precursor cells toward a mature osteoblastic phenotype. Ascorbic acid, a common constituent of the osteogenic medium, is a potent antioxidant that scavenges ROS and reduces oxidative stress [58]. Based on this, we postulate that our results may be attributed to the antioxidant activity of ascorbic acid, which helped maintain ROS levels within an optimal range for inducing osteogenesis in the MC3T3-E1 cells. We hypothesise that this balanced state may have supported the enhanced mineralisation observed following multiple CAP applications, thereby emphasising the importance of redox homeostasis in osteoblast differentiation. While we speculate that the redox balance mediated by ascorbic acid could represent the underlying mechanism behind our observed results, this remains a hypothesis that warrants experimental confirmation through future, more in-depth studies. Moreover, plasma composition and the generated RONS are critical in determining the stage-dependent responses of osteoblasts, as demonstrated by prior research. Using a low-temperature atmospheric nitrogen plasma, Prezkora *et al* [36] observed that short plasma exposures (4, 8, and 16 s) inhibited extracellular matrix mineralisation in MC3T3-E1 cells, even though the levels of the mineral-binding protein osteocalcin (OCN) were elevated. In contrast, Tominami *et al* [26] reported that cold atmospheric helium plasma treatment of MC3T3-E1 pre-osteoblasts not only enhanced early osteogenic markers but also promoted extracellular matrix mineralisation. These contradictory outcomes may be explained by the fact that helium and nitrogen plasmas generate distinct RONS profiles at different concentrations, which can differentially influence the stage-specific processes of osteoblast differentiation. However, future investigations focusing on the direct measurement of the intracellular ROS levels, antioxidant rescue experiments, and the analysis of plasma-activated medium, are required to clarify the

role of ROS-mediated signalling in the CAP-induced osteogenic differentiation.

4. Conclusions

The findings of this *in vitro* investigation highlight how CAP treatment modulates osteoblast viability and differentiation in an application- and time-dependent manner. Short, repeated CAP exposures (30 s–60 s) increased cell viability and proliferation rates, whereas longer exposures (90 s–120 s), particularly when applied over three consecutive days, significantly increased ALP activity, an early marker of osteogenesis, and promoted matrix mineralisation, an indicative of terminal differentiation of osteocytes from pre-osteoblasts. Taken together, these findings underscore the dual capacity of CAP to preserve cell function while stimulating osteogenic activity, suggesting a potential relevance for bone regeneration applications, including, possibly, alveolar bone regeneration. Nonetheless, certain limitations such as the lack of ROS quantification and the simplified *in vitro* model must be acknowledged. Specifically, the use of pre-osteoblastic MC3T3-E1 cell line, while widely accepted for studying osteogenic differentiation, does not fully represent the complex cellular composition or inflammatory environment of periodontal tissues. Therefore, the present results should be interpreted primarily within the context of basic osteogenic responses rather than the broader pathology of periodontitis. Additionally, owing to the fact that in the present study the investigations were conducted only on one cell type, e.g. MC3T3-E1 pre-osteoblasts, and no inflammatory or periodontal conditions were used as an experimental model, our findings should be viewed as preliminary and not yet representative of CAP behaviour in the diseased tissues. Moreover, further studies are required to elucidate the underlying molecular mechanisms, particularly those involving redox-signalling pathways, and to validate these effects in periodontal-specific cell types as well as *in vivo* models. Ultimately, only after a more comprehensive evaluation within complex microenvironments, the potential role of CAP as a therapeutic strategy can be properly assessed. If future research validates its efficacy, CAP could become a vital tool in oral medicine by offering a non-invasive, cell-responsive treatment modality for tissue regeneration and inflammation control.

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Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Supplementary Figure S1 available at https://doi.org/10.1088/2057-1976/ae744b/data1.

Supplementary Figure S2 available at https://doi.org/10.1088/2057-1976/ae744b/data2.

Conflict of interest

The authors declare no conflicts of interest.

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