Effect of different osteogenic media and saline solutions on the osteogenesis protocol using MC3T3-E1 subclones

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Abstract

Adequate laboratory protocols may improve the study of bone tissue and its metabolism. Thus, the use of effective techniques for staining bone cells in vitroand evaluating their function is significant. The cell line used for this purpose was MC3T3-E1, which contains preosteoblasts with mineralization potential. Several osteogenic media are used in the culture of these cells, and a variety of saline solutions are used for washing cultures in mineralization staining protocols and in situ alkaline phosphatase detection. Thus, the objective of the present study was to evaluate the effects of different osteogenic media in the culture of MC3T3-E1 cells, subclones 4 and 14, in addition to washing with Dulbecco's phosphate saline solution (DPBS) and Hanks' balanced salt solution (HBSS) in an alizarin red staining assay and situ alkaline phosphatase labeling via the Fast Red method. The cells were seeded at a density of $1x10^4$ cells/well for 7 and 10 days for the Fast Red assay and 12, 14, and 17 days for the staining of mineralization nodules. The data were statistically analyzed and significance was set for $p < 0.05$. Data obtained showed that the presence of dexamethasone significantly enhanced ALP detection in subclone 14 osteoblastic cells after 7 and 10 days as well as in subclone 4 cells washed with DPBS after 7 and 10 days when compared to control. Washing with Hanks' solution significantly increased the quantification of ALP at 10 days and of mineralized nodules in 4 subclone cells after 17 days. Moreover, alizarin red staining improved, resulting in a more intense red color, in the group that was washed with Hanks' solution for both subclones in all experimental periods. Thus, it is suggested that washing with Hanks' salt solution is better for in vitro staining of calcium nodules when using the alizarin red method.

Keywords: osteoblast, cell line, alizarin red, alkaline phosphatase, saline solutions.

Efeito de diferentes meios osteogênicos e soluções salinas no protocolo de osteogênese utilizando subclones MC3T3-E1

Resumo

Protocolos laboratoriais adequados podem melhorar o estudo do tecido ósseo e do seu metabolismo, sendo relevante o uso de técnicas eficazes para coloração de células ósseas *in vitro* e avaliação de sua função. A linhagem celular utilizada para esse fim foi a MC3T3-E1, pré-osteoblastos com potencial de mineralização. Vários meios osteogênicos são utilizados na cultura dessas células, e uma variedade de soluções salinas são utilizadas para lavagem de culturas em protocolos de coloração de mineralização e detecção *in situ* de fosfatase alcalina. Assim, o objetivo do presente estudo foi avaliar os efeitos de diferentes meios osteogênicos no cultivo de células MC3T3-E1, subclones 4 e 14, além da lavagem com solução salina fosfato de Dulbecco (DPBS) e solução salina balanceada de Hanks (HBSS) em um ensaio de coloração com vermelho de alizarina e marcação de fosfatase alcalina *in situ* através do método Fast Red. As células foram semeadas a uma densidade de $1x10^4$ células/poço durante 7 e 10 dias para o ensaio de Fast Red e durante 12, 14 e 17 dias para a coloração de nódulos de mineralização com

vermelho de alizarina, sendo os dados analisados estatisticamente com a significância estabelecida para $p < 0,05$. Os resultados mostraram que a presença de dexametasona aumentou significativamente a detecção de ALP nas células do subclone 14 após 7 e 10 dias, assim como nas células do subclone 4 lavadas com DPBS após 7 e 10 dias, quando comparado ao controle. A lavagem com a solução de Hanks aumentou significativamente a quantificação de ALP aos 10 dias e de nódulos mineralizados aos 17 dias nas células do subclone 4. Além disso, a coloração com alizarina red foi aprimorada, resultando em uma cor vermelha mais intensa, no grupo que foi lavado com a solução de Hanks para ambos os subclones em todos os períodos experimentais. Assim, sugere-se que a lavagem com a solução salina de Hanks seja mais eficaz para a coloração in vitro de nódulos de cálcio ao utilizar o método de coloração com vermelho de alizarina.

Palavras-chave: osteoblastos, linhagem celular, vermelho de alizarina, fosfatase alcalina, soluções salinas.

1. Introduction

Cell culture is currently an important tool for research that allows the discovery of different mechanisms of cell functioning, and the development of new techniques and *in vitro* treatments, which are essential as a pretest for *in vivo* studies. In this way, it is possible to cultivate primary cultured cells and immortalized lineage cells. In primary culture, cells are removed directly from the substrate and cultured to reach confluence. These cells are subcultured in a new flask, and this subculture is called a cell lineage (Alves et al., 2010; Carrel, 1912; Freshney, 1994). The study of bone metabolism is highly important since there are several diseases related to this tissue, such as dysplasia, infections, and chronic diseases such as osteoporosis.

Therefore, it is crucial to develop techniques for evaluating bone tissue and its metabolism to improve the visualization of bone cell function and the deposition of mineralized nodules. An immortalized cell line widely used in investigations related to bone metabolism is MC3T3-E1. This cell line is derived from preosteoblasts from the calvaria of newborn mice and has a culture morphology similar to fibroblasts. This cell line was cultivated, cloned, and inserted into several subclones, from which the most promising subclones were selected for their mineralization potential and expression of genes correlated with osteogenesis (Wang et al., 1999). There are several subclones, but the most commonly used are subclone 14 (ATCC®, CRL2594™, Manassas, VA, USA) and subclone 4 (ATCC®, CRL-2593™, Manassas, VA, US).

Subclone 14 was isolated and is widely used for the study of *in vitro* osteoblastic differentiation and ECM signaling, while subclone 4 has a high capacity for osteoblastic differentiation and mineralization in a medium supplemented with ascorbic acid, one of the main *in vitro* inducers of differentiation of this lineage (Izumiya et al., 2021). However, some authors have used dexamethasone for the differentiation of these cells, with or without ascorbic acid and beta-glycerophosphate (Li et al., 2019). Thus, there is no standardization in the protocols for the culture of these cells, resulting in each laboratory using its methods. Furthermore, the most commonly used *in vitro* methods for assessing osteogenesis are the *in situ* alkaline phosphatase enzyme detection assay and the assessment of mineralized nodule deposition using alizarin red staining.

Similar to the cell differentiation method involving supplementation of the culture medium, these tests differ in their washing protocol with saline solutions. Balanced saline solutions are isotonic buffer systems that maintain the structural and physiological integrity of cells in vitro by preserving pH and osmotic balance while providing water and essential ions to cells (Thermo Fisher Scientific Inc.). There are several types of saline solutions used for washing, including 1) phosphate-buffered Spirulina (PBS), 2) Dulbecco's phosphate-buffered saline (DPBS), and 3) Hanks' balanced salt solution (HBSS). The differences among these solutions lie in their composition, which varies based on the presence of sodium chloride, phosphate buffer, glucose, and sodium bicarbonate." Some authors wash the cells with PBS, while others wash them with Hanks' solution, highlighting the importance of establishing standard protocols for laboratory use.

Therefore, the objective of the present work was to evaluate different MC3T3-E1 subclones (14 and 4) subjected to various culture media supplemented with osteoblastic differentiation inducers and two types of washing utilized for alkaline phosphatase *in situ* detection and alizarin red staining protocols.

2. Materials and Methods

2.1 Cell culture and treatment protocol

Cells from the MC3T3-E1 cell line, subclone 14 (ATCC®, CRL2594™, Manassas, VA, USA), and subclone 4 (ATCC®, CRL-2593™, Manassas, VA, USA) were thawed and expanded in 75 cm² culture bottles (Merck KGaA,

Darmstadt, Germany) containing 15 mL of minimal essential medium (MEM) (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, where they were kept in incubators humidified at 37 °C, with 95% atmospheric air and 5% CO_2. The medium was changed every two days until the cells reached confluence, and the cells were then removed from the bottles and plated in 24-well plates at $1x10^4$ cells per well containing 1 mL of MEM supplemented according to each treatment protocol.

The groups were divided as follows: 1) the control group, without the addition of any osteogenic inducer to the culture medium; 2) the group with the addition of 1% dexamethasone to MEM; 3) the group with the addition of 1% dexamethasone and a 1% solution of ascorbic acid and beta glycerophosphate to MEM; and 4) the group with the addition of 1% ascorbic acid solution and beta glycerophosphate to MEM. The plates were kept in an incubator, and the culture medium was changed every two days until the experimental period was established.

2.2 In situ evaluation of alkaline phosphatase by the Fast Red method

In situ detection of alkaline phosphatase was performed after 7 and 10 days of culture. After removing the culture medium, the wells were washed twice with DPBS warmed to 37 °C. Five hundred sixty milligrams of Triz reagent (Sigma, Germany) was initially dissolved in 35 mL of deionized water, and 14 mg of Fast Red reagent (Sigma, Germany) was added. After discarding 3.5 mL of this solution and adding 12.25 mg of naphthol (Sigma, Germany) diluted in 3.5 mL of dimethyl formamide (Merck, Germany), the working solution was prepared. One milliliter of this solution was added to each well. The plate was incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 30 minutes. After this incubation, the solution was removed from the wells, and the plates were left to dry at room temperature for photography and subsequent qualitative analysis. The same protocol described above was also used, but the washing solution was replaced with Hanks' solution.

2.3 Alizarin Red S staining

The mineralized nodules were stained with alizarin red following the decalcification protocol described by Stanford et al. (1995) on days 12, 14, and 17. The medium was removed from the wells, and the cells were subsequently washed twice with DPBS and fixed in 70% ethanol for 1 hour at 4 ºC. After this period, the cells were removed from the refrigerator and washed with DPBS and deionized water. The wells were left to dry, and then 40 mM Alizarin Red solution was added for 15 min at room temperature. After this period, the wells were washed with deionized water and then with DPBS for 15 min. Then, the solution was completely removed, and the wells were left to dry at room temperature. The same protocol described above was also used; however, the DPBS wash was replaced with a Hanks solution wash for the other studied groups.

2.4 Alizarin Red quantification

For alizarin red staining quantification, following the method of Gregory et al. (2004), 150 µL of 10% acetic acid was added to each well, and the plates were left under gentle agitation for 30 min. The cell layer was then scraped off with a cell scraper, and the solution was transferred to 1.5 mL *Eppendorf* tubes, heated at 85 °C for 10 min, and subsequently transferred to ice for 5 min. The tubes were centrifuged at 13,000 rpm for 20 min. A volume of 100 µL of the supernatant was transferred to a 96-well plate (Corning), and 40 µL of 10% ammonium hydroxide (Quimibras, Rio de Janeiro, RJ, Brazil) was added to each well. The absorbance was measured using a spectrophotometer (Bio-Tek) at a wavelength of 405 nm.

3. Results

3.1 Morphological evaluation of MC3T3-E1 cells in cell culture

After 4 days of culture, the cells were photographed at 10x magnification under an inverted light microscope (Eclipse Ti-S®, Nikon, Tokyo) to assess the morphological changes that occurred based on the type of osteogenic medium used, as shown in (Figure 1). After 4 days of culture, the cells in all groups exhibited a more rounded morphology, indicating that they were undergoing initial differentiation. A morphological difference between the cells from subclones 4 and 14 was observed (Figure 1). In subclone 4, the use of ascorbic acid and beta glycerophosphate resulted in an elongated cell morphology, similar to that of fibroblasts. In subclone 14, all the groups exhibited greater proliferation than subclone 14 cells, except for the group treated only with ascorbic acid and beta-glycerophosphate, exhibiting more rounded cells.

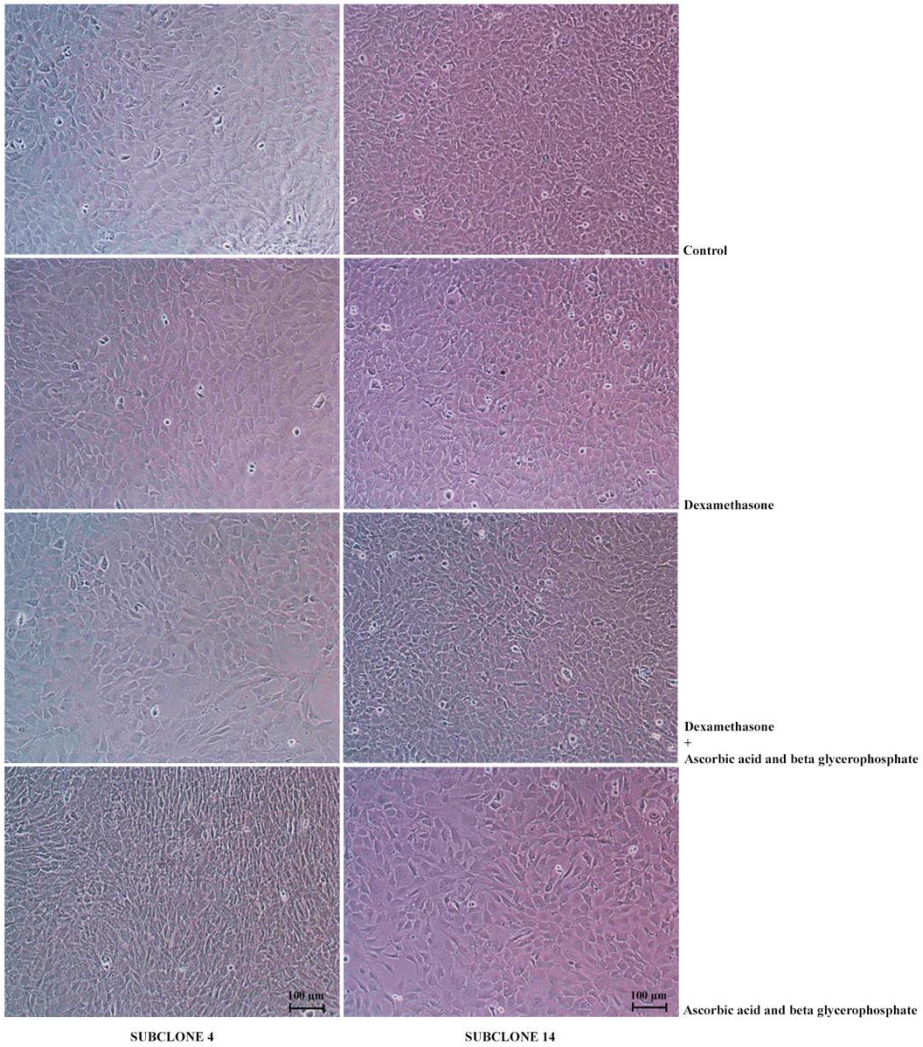

Figure 1. MC3T3-E1 cells in culture, 10x magnification. Column 1 represents cells from subclone 4 and column 2 represents cells from subclone 14. Each row represents the different osteogenic media used. Source: Authors, 2024.

3.2 In situ evaluation of alkaline phosphatase by the Fast Red method

The *in situ* deposition of the enzyme alkaline phosphatase was evaluated on days 7 and 10. The plates were photographed and quantified with ImageJ software, and the results are presented in (Figures 2 and 3) for subclones 14 and 4, respectively. Subclone 14 showed similar behavior during both periods, as shown in Figure 2. Both cells washed with DPBS and those washed with Hanks' solution exhibited greater alkaline phosphatase activity in the groups treated with dexamethasone alone and associated with ascorbic acid and beta-glycerophosphate at 7 and 10 days, with the values being higher in the latter period.

This difference was statistically significant when compared to the groups not treated with dexamethasone ($p < 0.05$). Besides, the utilization of different washing solutions did not affect the results of the *in situ* alkaline phosphatase quantification for subclone 14 osteoblastic cells. Figure 3, in contrast, shows the quantification of the *in situ* deposition of alkaline phosphatase in subclone 4 cells. In this situation, dexamethasone-induced a greater deposition of alkaline phosphatase at both 7 and 10 days when compared to the control group, except for the group washed with Hanks' solution at 10 days. Washing the cultures with Hank's solution affected the quantification results, decreasing ALP detection when compared to DPBS washing, especially for the group with dexamethasone. On the other hand, after 10 days, the quantification was increased for all the groups washed with Hanks' solution.

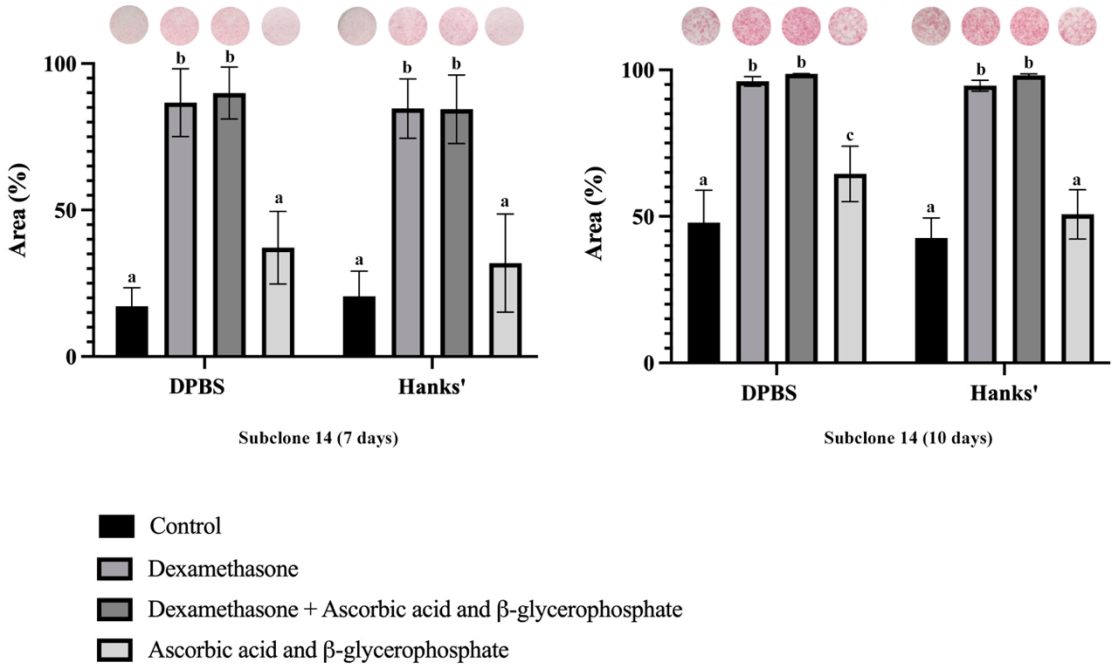

Figure 2. *In situ* quantification of alkaline phosphatase enzymes (percentage/area), MC3T3-E1 – subclone 14. Periods of 7 and 10 days. Different lowercase letters indicate significant differences at the 5% level. One-way ANOVA statistical test followed by Tukey's multiple comparisons test. Source: Authors, 2024.

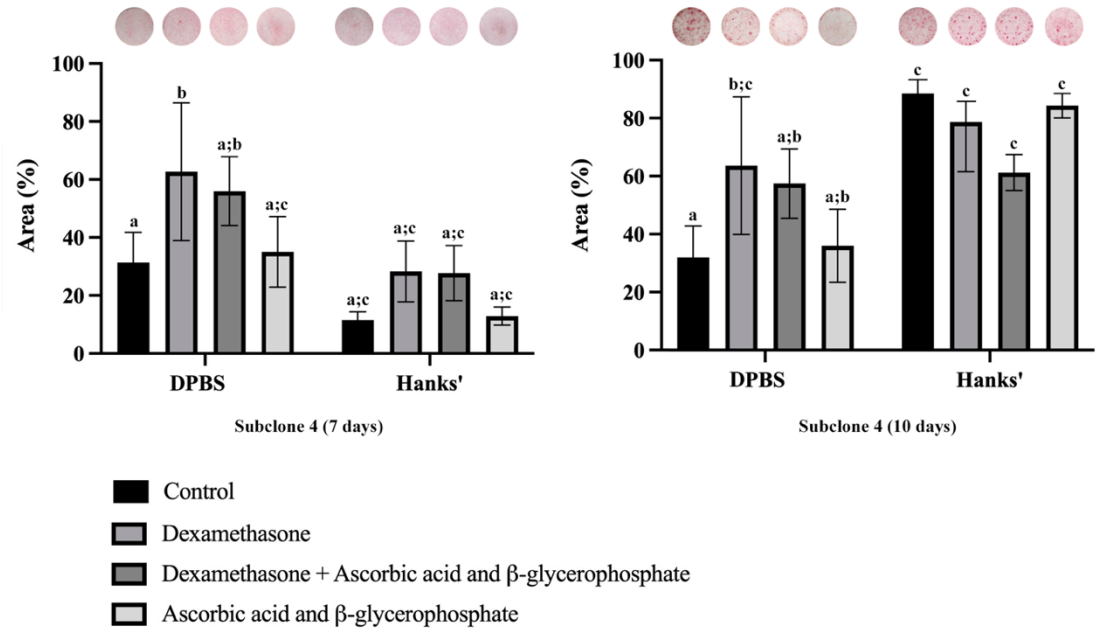

Figure 3. *In situ* quantification of alkaline phosphatase enzymes (percentage/area), MC3T3-E1 – subclone 4. Periods of 7 and 10 days. Different lowercase letters indicate significant differences at the 5% level. One-way ANOVA statistical test followed by Tukey's multiple comparisons test. Source: Authors, 2024.

3.2 Alizarin red quantification

Mineralized nodule deposition was evaluated on days 12, 14, and 17 of culture. Figure 4 presents images of mineralized nodules stained with Alizarin Red S, washed with DPBS and Hanks' solution. The deposition of nodules was comparable in subclones 4 and 14, with the most significant difference related to the type of saline solution used in the washing protocol. For both subclones, the nodules washed with Hanks' solution displayed brighter and clearer red staining compared to those washed with DPBS. Washing with DPBS produced nodules that appeared closer to brown on day 12 and more orange on days 14 and 17. In contrast, washing with Hanks' solution resulted in colors that varied from dark pink to red, and then to an intense wine color at days 12, 14, and 17, respectively.

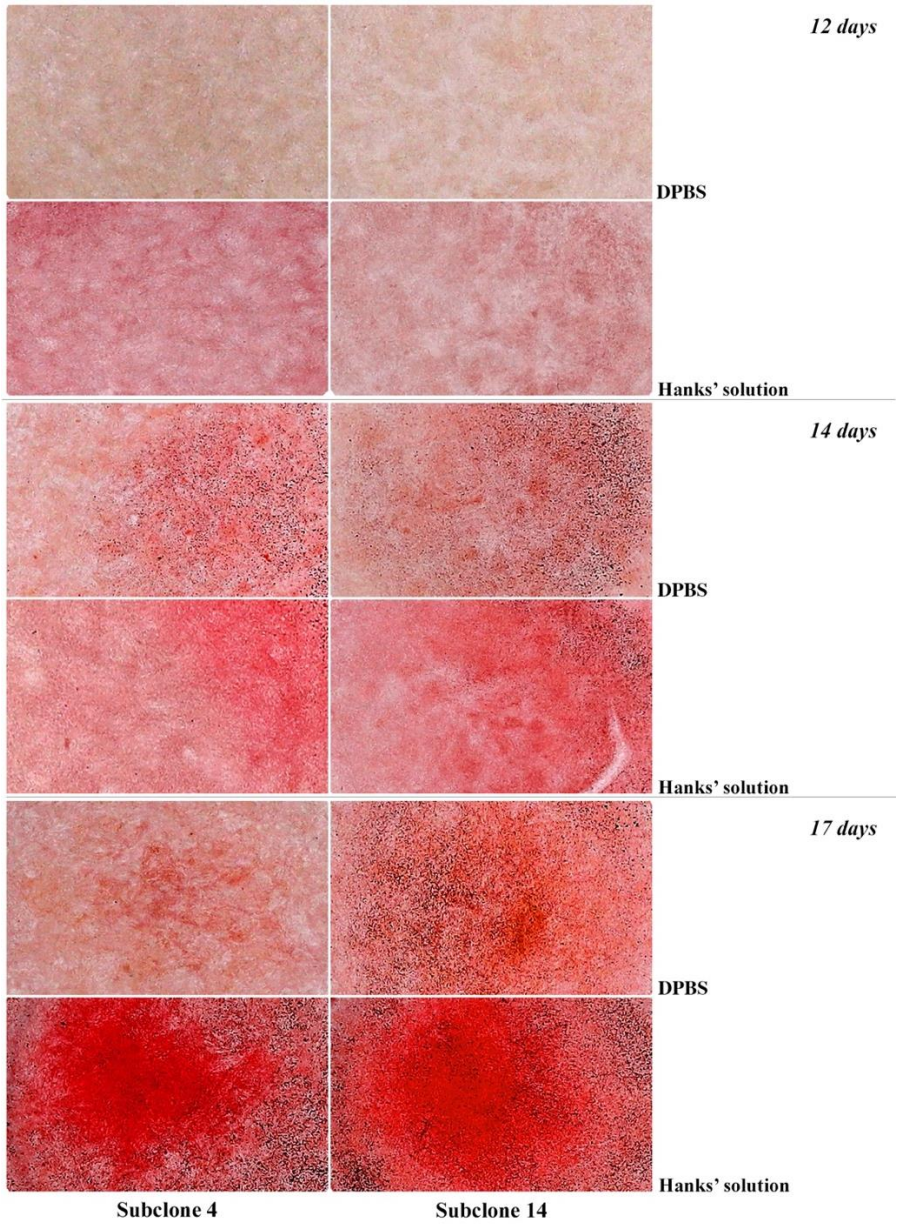

Figure 4. Images of mineralization nodules stained with alizarin red and subclones 4 and 14. Source: Authors, 2024.

Figures 5 and 6 illustrate the quantification of alizarin red extraction from subclones 14 and 4, respectively, measured by absorbance at 405 nm. For both subclones, nodule deposition increased progressively over time and was higher in the group washed with Hanks' solution. It is interesting to observe that the group washed with DPBS

and treated with ascorbic acid and beta-glycerophosphate exhibited significantly lower deposition compared to the other groups ($p < 0.05$). For the other time points, there was no significant difference among the groups analyzed for subclone 14. Conversely, the data shown in Figure 6 for subclone 4 revealed that washing with Hanks' solution after 12 days was more effective for cells treated with ascorbic acid and beta-glycerophosphate when compared to the other groups ($p < 0.05$). At 14 days, no significant differences were observed; however, at 17 days, mineralization in all the groups washed with Hanks' solution was significantly higher when compared to the groups washed with DPBS ($p < 0.05$).

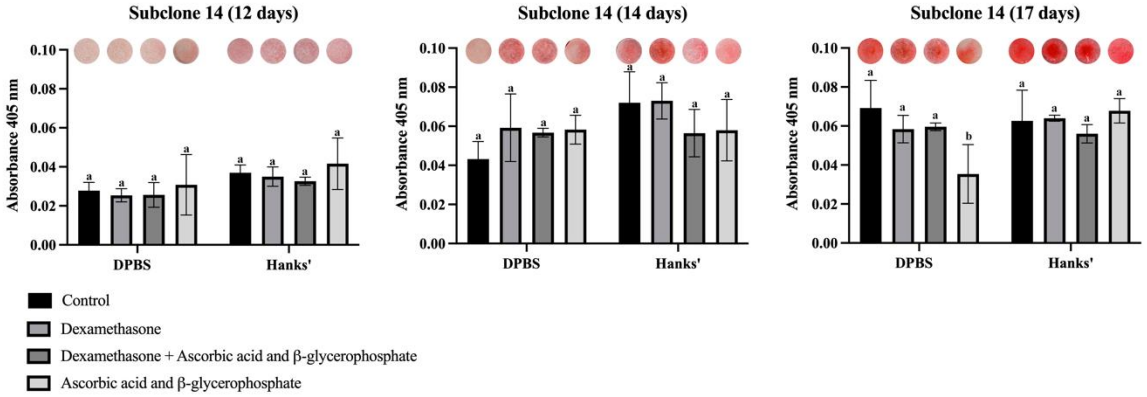

Figure 5. Quantification of mineralized nodules of the MC3T3-E1 strain subclone 14 at 12, 14, and 17 days. Different lowercase letters indicate significant differences at the 5% level. One-way ANOVA statistical test followed by Tukey's multiple comparisons test. Source: Authors, 2024.

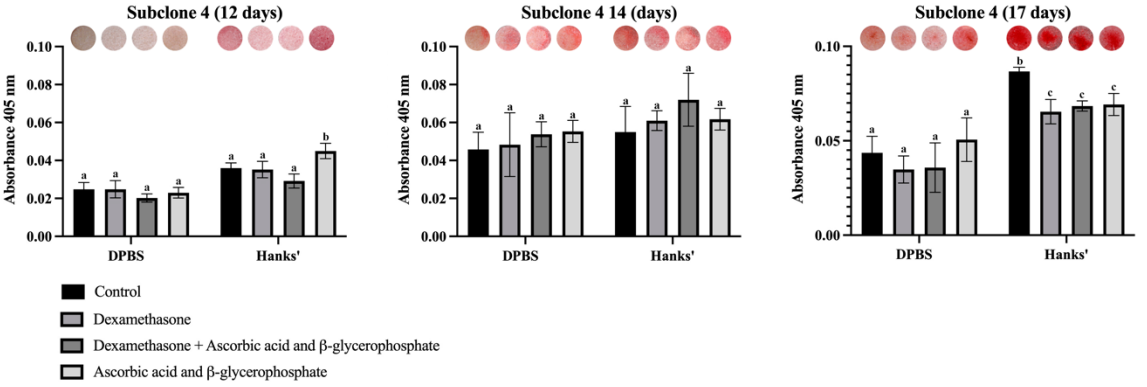

Figure 6. Quantification of mineralized nodules of the MC3T3-E1 strain subclone 4 at 12, 14, and 17 days. Different lowercase letters indicate significant differences at the 5% level. One-way ANOVA statistical test followed by Tukey's multiple comparisons test. Source: Authors, 2024.

4. Discussion

The osteogenic differentiation in MC3T3-E1 subclones involves an initial active proliferative stage without expressing markers of mature osteoblasts, such as alkaline phosphatase or mineralized matrix. This stage is followed by growth arrest to establish differentiation, marked by the expression of alkaline phosphatase activity and collagen matrix deposition (Quarles et al., 1992; Suzuki et al., 2019). Subclonal variations in MC3T3-E1 cells can lead to differences in the mineralization potential.

Some subclones exhibit high mineralization potential and express osteoblast markers such as bone sialoprotein and osteocalcin, while others do not, despite similar levels of alkaline phosphatase expression (Wang et al., 1999). Hwang et al. (2019) showed the variability among MC3T3-E1 subclones, noting that subclone 4 was responsive to parathyroid hormone (PTH) stimulation and capable of matrix mineralization, whereas subclones 14 and 24 did not replicate these key aspects of osteoblast biology. Thus, the variability in function and morphology among

different MC3T3-E1 subclones, leading to variable osteogenic performance, impacts their utilization in cell culture. Regarding the different osteogenic media utilized for cell culture in the present investigation, it was observed that cell morphology and proliferation were different in each subclone, especially in contact with the ascorbic acid and beta-glycerophosphate association, suggesting that the composition of media may interfere differently in each subclone cell behavior.

According to Quarles et al. (1992), MC3T3-E1 subclones 4 and 14 exhibit a fusiform morphology during the proliferative phase and transition to a cuboidal morphology during the differentiation phase in osteogenic media containing ascorbate and beta-glycerol phosphate, with our data suggesting that cells from subclone 14 submitted to media with ascorbic acid and beta-glycerophosphate were already undergoing differentiation as early as 4 days of culture. For ALP in situ quantification, it can be inferred that the presence of dexamethasone is important to promote ALP deposition for both subclones, but especially and significatively for subclone 4.

Previous reports have already shown that dexamethasone plays a significant role in modulating alkaline phosphatase (ALP) activity, such as the study conducted by Cheng et al. (1996), who observed a significant increase in ALP activity for 23 days in human bone marrow stromal cells. Regarding the formation of mineralized nodules, in the present study, there were no differences among the different media used for both subclones, even though mineralization increased along the time of the culture.

Another issue investigated in this study was the evaluation of two types of washing solutions (DPBS and Hanks' solution) for alkaline phosphatase in situ detection and alizarin red staining protocols in both 4 and 14 subclone cultures. A variety of substances can be employed to achieve this outcome, like simulated body fluid (SBF), commonly used in biomimetic mineralization studies (Fu et al., 2024), Dulbecco's phosphate-buffered saline (DPBS), used to maintain the pH and osmotic balance (Petrakova et al., 2021) and Hank's balanced salt solution (HBSS), used for washing and maintaining cells in a physiological state (Zhang et al., 2016). The use of washing solutions in these protocols is particularly important to ensure the removal of unwanted residues, the proper maintenance of the mineralizing environment, and the consistency of results.

In the present investigation, DPBS and Hank's solution were utilized for the experiments. DPBS is a simpler solution that contains phosphate buffer, while Hanks' solution is more complex and contains 0.350 g/L^{-1} sodium bicarbonate. Table 1 presents the main differences in composition between each of these saline solutions. The presence of inorganic ions, glucose, and phenol red in Hanks' solution stands out, whereas they are not present in DPBS. According to the manufacturer, inorganic salts and glucose are essential for normal cellular metabolism (Hanks, 1976; Hanks; Wallace, 1949).

Effectively, the present results showed an improvement of ALP in situ and mineralized nodules quantification, especially in later culture periods of subclone 4 cells after washing with Hanks' solution. Besides, there was an enhancement in alizarin red staining, which suggests an interaction between the alizarin red S dye and the washing medium that changes the final color staining. After removing the dye, the color changed with the use of the saline solution during the 15-minute wash in the mineralization protocol described in section 2.3, corroborating this suggestion. Alizarin red S is a dye that binds to calcium by a chelation process, coloring calcium salts red, and is widely used for marking and quantifying mineralized nodules in situ (Wu; Forsling, 1993; Puchtler et al., 1969).

According to Lemlikchi et al. (2014), the addition of phosphate causes a pH change due to the formation of complexes that increase the acidity of alizarin, and this dye, in turn, undergoes a color change according to the pH. The authors reported that in a pH range of 7 to 8, the color of alizarin becomes pink, while in a pH range of 5.43 to 5.8, alizarin acquires a reddish brown color.

When we measured the pH of Hanks' solution and DPBS after washing, we observed that the pH of DPBS decreased from 7.0 to 5.79, while the pH of Hanks' solution remained at 7.0. Thus, this change in the pH of the solution explains the final color of the stained nodules observed in Figure 4, especially at 12 days of culture, which indicates that the type of saline solution used in the washing of the mineralization protocol influences the final color of the dye in the staining of calcium nodules. Even though this investigation provides useful insights into the variability of osteogenic differentiation across MC3T3-E1 subclones and the impact of washing solutions on mineralization assays, some limitations can be mentioned, such as the limited scope of washing solutions tested and the absence of molecular assays to observe pathways by which the different washing solutions affect staining outcomes.

Table 1. Composition of the saline solutions used in the washings of the experiments carried out.

Solution	DPBS	Hanks'
Composition	• Potassium chloride • Monobasic phosphate chloride • Sodium chloride • Dibasic Sodium Phosphate Anhydrous	• Calcium chloride dihydrate • Anhydrous magnesium sulfate • Potassium chloride • Sodium chloride • Sodium hydrogen phosphate anhydrous • Monopotassium phosphate • *D*-glucose • Phenol Red

Source: Authors, 2024.

5. Conclusions

The data were statistically analyzed and significance was set for $p < 0.05$. Data obtained showed that the presence of dexamethasone significantly enhanced ALP detection in both subclones. Besides, washing with Hanks' solution significantly increased the quantification of ALP in situ and mineralized nodules in subclone 4 cells in a later period of the culture as well as improved alizarin red staining in both subclones in all experimental periods. Thus, it is suggested that washing with Hanks' salt solution is a better choice for alkaline phosphatase *in situ* detection and alizarin red staining protocols.

6. Acknowledgements

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7. Authors' Contributions

Maria Carolina Coelho: received the FAPESP scholarship to perform the project. She performed all the experiments and helped with manuscript writing. *Luiz Gabriel Plasier Lazari Guedes de Melo*: helped to plan the experiments and with cell line proliferation to be used the all experimental groups. *Sayuri Poli Suguimoto*: helped with the cell culture biochemical assays and data analysis. *Roger Rodrigo Fernandes*: is the cell culture lab technician and supervises all the experiments performed by the students. *Karina Fittipaldi Bombonato-Padro*: is the supervisor of the project, obtained financial support, supervised the experiments, analyzed the results, and helped write the manuscript.

8. Conflicts of Interest

The authors declare that they have no conflicts of interest.

9. Ethics Approval

Not applicable.

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