

Osteogenic Behaviour of Human Mesenchymal Stem Cells to Injection-Moulded Porous NiTi Implants and Nickel Ion Release

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Abstract

Objective

This study evaluated the osteogenic potential of porous NiTi dental implants fabricated via metal injection moulding (MIM) and investigated the associated nickel ion release behaviour.

Methods

Human mesenchymal stem cells (hMSCs) were cultured on porous NiTi implants, machined NiTi, and Ti6Al4V surfaces. Cell proliferation, alkaline phosphatase activity, and mineralisation were assessed at predetermined time points. Nickel ion release was quantified over 21 days.

Results

Porous NiTi surfaces demonstrated significantly enhanced cell proliferation, and mineralised matrix formation compared with machined NiTi and Ti6Al4V controls ($p < 0.05$). Whereas ALP activity was higher in porous NiTi, but not statistically different. Nickel ion release showed a transient increase during the early culture period, peaking on day 14 and then declining.

Conclusion

Porous NiTi implants fabricated via MIM support osteogenic differentiation of hMSCs while exhibiting controlled, transient nickel ion release. These findings suggest that porous NiTi structures may provide a favourable surface environment for bone integration and hold promise as alternative biomaterials for dental implant applications.

1.0 Introduction

Tooth loss is a major global health concern, affecting both oral health and overall quality of life [1]. Fortunately, dental implants have emerged as the preferred solution for replacing missing teeth, known for their high reliability and successful outcomes [2].

Advances in implant surface design have increased the 10-year survival rate from 89% to 97% [2]. However, factors such as diabetes, osteoporosis, smoking, and periodontitis can reduce success rates [3–5]. Thus, researchers continue to seek solutions. Titanium and its alloys remain the primary materials for dental implants. However, their high Young's modulus (110 GPa), which greatly exceeds that of cortical and cancellous bone (10–30 GPa), can lead to stress shielding. β -type titanium alloys such as Ti35Nb4Sn have a lower elastic modulus (~ 80 GPa), but research is still ongoing [6]. To mitigate the modulus mismatch, scientists are investigating low-elastic-modulus coatings, despite manufacturing

and interfacial challenges [7]. Since the 1990s, nickel-titanium (NiTi) has attracted interest in biomedical fields because of its superelasticity and a modulus closer to that of cortical bone. Nonetheless, complex manufacturing processes have limited its use mainly to simple orthopaedic devices, such as staples, and to cardiovascular devices such as stents [8, 9]. Recent developments in powder metallurgy and additive manufacturing aim to overcome these obstacles, enabling improved uniformity and the fabrication of complex designs. Additive manufacturing faces issues such as surface finish and geometric constraints [10]. Meanwhile, powder metallurgy methods such as metal injection moulding (MIM) and reactive sintering address concerns such as intermetallic formation and impurities, enabling more homogeneous materials and complex structures, including porous designs [11]. These technological advances have demonstrated promising biocompatibility in prior *in vivo* and animal tests [12–13].

The surface characteristics of implants, such as roughness and topography, influence osteoblastic activity, including adhesion, growth, and differentiation, thereby affecting osseointegration [14–16]. Macro- and micrometer-scale roughness enhance mechanical stability, while nanometer-scale features promote protein adsorption [15]. Meanwhile, porous surfaces facilitate fluid flow during early osseointegration, supporting bone integration [17]. *In vitro*, surface topography impacts the proliferation and differentiation of various cells, like mesenchymal and osteoblast-like cells that display mature osteoblast traits [14, 18]. For instance, MG63 osteosarcoma cells show reduced proliferation but increased differentiation on rougher surfaces, evidenced by higher levels of alkaline phosphatase and osteocalcin compared to smoother surfaces [19].

While the mechanical benefits of NiTi are notable, the release of nickel ions raises concerns about cytotoxicity and allergic reactions. Nevertheless, the strong interatomic bonds and crystal lattice of the equiatomic NiTi alloy help minimise ion leaching [21]. Furthermore, titanium has a greater tendency to oxidise, forming a protective TiO₂ layer that prevents corrosion and slows nickel-ion diffusion. Nonetheless, nickel ions in this oxide layer may still influence ionic motion [21]. Research shows that even at subtoxic levels, nickel can affect osteoblast activity, decreasing alkaline phosphatase (ALP) levels and DNA synthesis [22]. The relationship between cellular responses to porous NiTi fabricated through metal injection molding (MIM) and the potential release of nickel ions from these structures has not been extensively investigated. To the author's knowledge, no research has examined the biological response of human mesenchymal stem cells (hMSCs) to porous NiTi dental implants fabricated via MIM. Therefore, the aim of this study was to evaluate the osteogenic potential of porous NiTi dental implants fabricated by MIM using hMSCs and to investigate the associated nickel-ion release behaviour over time.

2.0 Material and Methods

2.1 Sample preparation and study design

The sample was prepared as in previous studies [23, 24]. Using the MIM process, a 4.5 mm-diameter, 10 mm-long dental implant was fabricated from nickel-titanium with a porous surface topography (porous NiTi) ($S_a = 8.998 \mu\text{m}$); the average pore size was $50 \mu\text{m}$, with 20–30% porosity. The fabrication process involved several steps, including injection moulding, solvent and thermal debinding, and sintering, which took place at a certified facility (Nitium Technology Sdn. Bhd, Selangor, Malaysia, ISO 13485:2016) based on prior research. The feedstock consisted of a blend of nickel (Ni) and titanium (Ti) powders, accounting for 67.50% of the total volume. The remaining feedstock volume consisted of polymer binders. 56:44 wt % of the nickel/titanium ratio was utilized to achieve pseudoelasticity. The fabricated implant had an average pore diameter of $50 \mu\text{m}$ and a porosity of 25–30%. The surface roughness is formed due to binary interactions between the binder's molecular chain. The water-soluble binder helped feedstock flow during the injection moulding process, while the backbone binder provided rigidity to the moulded item. Additionally, the surfactant prevented powder aggregation. During the heat debinding process, the backbone binder densified, creating an interconnected porous structure and interparticle linkages. The similar surface-machined Ti6Al4V ($S_a = 5.12 \mu\text{m}$) and the machined-surface NiTi fabricated by casting ($S_a = 6.99 \mu\text{m}$) were used as positive controls; both were provided by the same company in this study.

Samples for each biomaterial group were evenly distributed across predetermined evaluation time points on days 1, 7, 14, 21, and 28 after seeding ($n = 6$ per predetermined time point, with replicates). The respective media were collected simultaneously at the predetermined times for the nickel (Ni) ion release study. To evaluate the effect of pH in 0.9% NaCl on Ni ion release, a total of 12 samples ($n = 3$ per pH group) were prepared and evaluated on days 1 and 3 (immediate), day 14 (intermediate), and day 28 (late).

2.2 Monoculture with Human Mesenchymal Cells

As this is solely an *in vitro* study, commercially obtained de-identified poietic™ human mesenchymal stem cells (hMSCs; PT-2501, Batch no: 22TL301024), sourced from Lonza Walkersville Inc., Walkersville, MD, USA, were used. Ethical approval was not required because no identifiable human participants or animals were directly involved in this research.

2.2.1 Initiation of Cell Culture and Seeding on Biomaterial

Poietic™ human mesenchymal stem cells (hMSCs; PT-2501, Batch no: 22TL301024) were purchased from Lonza (Walkersville Inc., Walkersville, MD, USA). The cells were received at passage 2 and were donated by a healthy 23-year-old male donor. Cell thawing and subsequent subculture strictly adhered to Lonza's technical instructions until passage 5. Briefly, the thawed cell suspension was added to a 15 mL centrifuge tube containing 5 mL of temperature-equilibrated medium using a micropipette. The tube was then centrifuged at $500 \times g$ for 5 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended with 1 mL of temperature-equilibrated human mesenchymal stem cell growth medium (MSCGM™, Lonza Walkersville Inc, Walkersville, MD, USA) by gently pipetting up and down. Total cell viability was assessed (95%), and the volume of suspended cells required for each 25 cm² flask

containing 5 mL of MSCGM™ was calculated (6×10^3 cells per cm²). The flask was gently rocked after seeding to disperse the cells evenly, then incubated at 37°C with 5% CO₂ and 90% humidity. The culture medium was replaced every 3 days until the cells reached nearly 90% confluence, at which point they were ready for subculture.

In passage 5, 50 µL of MSCGM™ medium containing 1.5×10^4 cells was carefully added to each sterile biomaterial (positive control: machined surfaces of NiTi (mNiTi) fabricated by a conventional method and Ti6AL4V, Ti) and to the test dental implant (porous NiTi Dent Tuah dental implant) that had already been placed in a 24-well plate. After 15 minutes of incubation of the seeded hMSCs on the biomaterials in a humidified atmosphere with 5% CO₂ at 37°C to allow initial cell attachment, 950 µL of osteogenic medium was added.¹⁸ The osteogenic medium (OM) responsible for inducing osteogenic differentiation of these cells comprises Dulbecco's Modified Essential Medium (DMEM, Sigma-Aldrich), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 units/ml penicillin-streptomycin (Gibco), 50 nM dexamethasone, 0.2 mM ascorbic acid and 10 mM β-glycerophosphate. In another 24-well plate, 1.5×10^4 cells were suspended in 1 mL osteogenic medium as a negative control. After 1 day of incubation, all biomaterials were transferred to a new well in the plate where the negative control was suspended. Fresh OM was added (1 mL for each well) and replaced every 3 days.

2.2.2 Cell Viability Assay

The cellular metabolic activity was determined by identifying cell viability using colorimetric assay, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide), (MTT Merck, Germany). As a result of dehydrogenase activity by mitochondria of the viable cells on this reagent, the pale-yellow tetrazolium salt of MTT colour will yield a purple formazan crystal. Following the manufacturer's instructions, 100 µL MTT mixture was added to each cell-seeded well for controls and NiTi dental implants. The plate was gently tapped on the side of the tray to mix the mixture, covered with aluminium foil, and then incubated for 4 hours (5% CO₂, 37°C). Next, 0.04 N HCl in isopropanol was added to each well and mixed thoroughly. Absorbance was immediately measured using an ELISA plate reader at 570 nm. The assay was performed in replicates on days 1, 7, 14, 21 and 28.

2.2.3 Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was measured in this study assessing osteogenic differentiation of hMSCs in osteogenic media using an ALP assay (MAK 447, Merck KGaA, Darmstadt, Germany). Activity was assessed at 7 and 14 days post-seeding. The cells were washed twice with 1 mL of PBS. The cells were then lysed by shaking for 20 minutes in 0.5 mL of 0.2% Triton X-100 (Sigma-Aldrich) in purified water at room temperature. Then, 50 µL of each sample was transferred into a 96-well plate containing 150 µL of working reagent, in triplicate. The working reagent comprises a mixture of assay buffer, 0.2 M Mg acetate, and 1 M p-nitrophenol phosphate solution prepared earlier, following the manufacturer's guidelines. At the same time, 200 µL of purified water and 200 µL of calibrator liquid were transferred into separate wells of the 96-well plate. The plate was tapped gently to ensure the mixture was well mixed.

Immediately, the optical density (OD) of each prepared mixture was measured on a plate reader at 405 nm ($T = 0$ minutes), and the measurement was repeated after 4 minutes ($T = 4$ minutes).

2.2.4 Alizarin Red S staining

Calcium deposition, indicating mineralization in this monolayer cell culture, was assessed using Alizarin Red S assay (ScienCell's ARS Staining Quantification Assay, Ared-Q, catalogue #8678). Following 21 and 28 post-seeding, the culture medium from each well was removed, and the cells were gently rinsed three times with 1 ml phosphate-buffered saline (PBS). The cells were then fixed in 4% formaldehyde for 15 minutes at room temperature. Next, the fixative was aspirated, and the container was rinsed three times with distilled water. Once the distilled water was completely removed, 1 mL of 40 mM ARS was added to each well, and the plate was incubated at room temperature with gentle shaking at 40 rpm for 20–30 minutes. Then, the dye was removed, followed by rinsing of the cell (five times) with distilled water. The plate was tilted at an angle of approximately 2° for 2 minutes to facilitate the removal of excess water, then stored at -20°C before dye extraction. Quantification of mineralization was done by acid extraction of the ARS stain following the protocol recommended by the manufacturer.

200 μL of 10% acetic acid was added to each well, followed by incubation at room temperature with gentle shaking for 30 minutes. Cell monolayers on the negative, including the other samples (positive control and NiTi), are now loosely attached. The samples were transferred into a 50 mL centrifuge tube and vortexed for 30 seconds. Each tube was then sealed with Parafilm, heated at 85°C for 10 minutes, and transferred to ice for 5 minutes. The slurry was then centrifuged at 20,000 g for 15 minutes. After that, 200 μL of supernatant was transferred to a new tube. 75 μL of 10% ammonium hydroxide (pH 4.1–4.5) was added to neutralize the acid. Then, 50 μL of each sample was transferred into each well of a 96-well plate in triplicate. The absorbance of aliquots was measured at 405 nm with a plate reader.

2.2.5 Visualization of The Cultured Cells on Biomaterials

At predetermined points on days 7, 14, 21, and 28, the samples were collected and initially fixed in a 4% glutaraldehyde solution for 24 hours at 4°C . Subsequently, the samples were washed with 0.1 M sodium cacodylate buffer, pH 7.2, and then post-fixed in 1% Osmium Tetroxide for 2 hours. Once again, the wash step was repeated with a similar solution, and a series of dehydrations was performed in graded acetone solutions, ranging from 35% to 100%. Next, samples were infiltrated with an acetone-HMDS mixture, then dried, mounted on a stub, sputter-coated with platinum, and viewed under a scanning electron microscope (SEM; FEI Quanta 200) operated at 15 kV.

2.3 Nickel Ion Release

Nickel ion release was evaluated in three solutions, namely osteogenic media collected from machined-surface NiTi (mNiTi) and porous NiTi (pNiTi) dental implants at days 1, 3, 7, 14, 21 and 28 post-seeding, and 0.9% NaCl at pH 6.0 (± 0.2) and 7.0 (± 0.2). For both pH conditions, the NaCl solutions were stored at 37°C , and ion release was evaluated on days 1 and 3 (immediate), day 14 (intermediate), and day 28

(late). The nickel ion concentration was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 2100DV-Perkin Elmer).

2.4 Statistical Analysis

All datasets were analysed using SPSS 29.0 (IBM, Armonk, NY). A repeated-measures ANOVA was applied to all dependent variables. Pairwise comparisons with Bonferroni correction were conducted to detect significant differences from the prescribed treatment, subjects, and treatment-time interaction. If differences are identified among the variances, the statistical significance is set at $p < 0.05$.

3.0 Result

3.1 Human Mesenchymal Cells (MSCs) Monoculture

3.1.1 hMSCs Viability Assay

The viability of hMSCs was assessed using the MTT assay, a colourimetric method that quantifies cellular activity by measuring the absorbance of the culture medium. The assay is based on the reduction of yellow-coloured tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to purplish-coloured formazan crystals by active mitochondria in living cells. The mean optical density (OD) of hMSCs cultured on different biomaterials, namely machined surface Ti6AL4V (Ti), machined surface Nickel-Titanium (mNiTi), porous surface Nickel-Titanium (pNiTi), and a negative control (without any biomaterial) in osteogenic medium, is presented in Fig. 1. Cells were viable in response to the samples and proliferated across all biomaterial groups within 28 days of incubation. The negative, Ti, and mNiTi groups exhibited a similar pattern of increasing OD over the first 7 days of observation. Although the pNiTi OD was higher than in the other groups, it was slightly lower on days 7 and 14. Throughout the 28 days of incubation, pNiTi cells continued to proliferate from day 21 onwards. Similarly, the other groups' OD was slightly lower on day 7 and then increased through day 28. A repeated-measures ANOVA within groups was conducted and showed significant differences in OD over time ($F = 11.74$ (4, 11.74), $p < 0.01$). Following pairwise comparison with Bonferroni correction and confidence interval adjustment, significant differences were found between the negative group on day 1 and day 28 (-0.282 (-0.535 , -0.29), $p = 0.031$). Significant increases in cell viability were observed in several comparisons: Day 1 vs Day 7, Day 1 vs Day 21, Day 1 vs Day 28, and Day 14 vs Day 28.

Furthermore, the impact of various biomaterials on cell viability was notable. At all-time points, the average optical density values differed significantly among the biomaterials ($p < 0.001$). Post hoc analysis showed that the mean optical density of the negative control was considerably lower than that of all three biomaterials tested. Additionally, significant differences were observed between pairs such as Ti versus mNiTi, Ti versus pNiTi, and mNiTi versus pNiTi ($p < 0.001$).

3.1.2 ALP activity

Alkaline phosphatase (ALP) is a key bone marker associated with osteoblast activity during osteoblast proliferation, differentiation, and osteogenesis. This study evaluated ALP as an early indicator of mesenchymal stem cell (MSC) differentiation into the osteoblast phenotype, comparing enzyme activity on days 7 and 14.

As shown in Fig. 3, ALP activity on day 14 was significantly higher than on day 7, regardless of sample type ($F = 5.63$ (1), $p = 0.028$). However, across all sample groups, mean ALP levels did not differ significantly at either time point ($F = 2.425$ (3), $p = 0.096$). On day 7, the negative and pNiTi groups had nearly identical ALP levels, with means of 36.18 ± 15.22 and 36.27 ± 18.85 , respectively. By contrast, the mNiTi group had the lowest mean ALP level (25.55 ± 4.29). By day 14, ALP levels had increased in all groups, with the pNiTi group showing approximately a 20% rise in activity.

3.1.3 Alizarin Red S Staining

The investigation of calcium hydroxyl apatite deposition as an indicator of bone mineralization was performed using the Alizarin Red S Staining Assay (ARS). Mineralization was evaluated on days 21 and 28 of the study. As shown in Fig. 4, the absorbance values of the Alizarin Red S staining consistently increased over time, with day 28 recording the highest absorbance across all sample groups ($F = 83.595$ (1), $p < 0.001$). Furthermore, significant differences in absorbance values were observed among the various groups ($F(df) = 136.54$ (3), $p < 0.001$). Significant differences were found between the pNiTi group and the other groups ($p < 0.001$), as well as between the Ti and mNiTi groups ($p < 0.05$). Overall, all samples showed a steady increase in absorbance, indicating increased bone matrix deposition over time.

3.1.4 Scanning Electron Microscopy (SEM)

Figure 5 shows scanning electron images of hMSCs incubated on a glass slide (negative control), Ti, mNiTi, and pNiTi surfaces in osteogenic media. These observations highlight differences in cellular response and osteogenic differentiation influenced by substrate surface characteristics. On day 7, the cells displayed elongated processes, suggesting differentiation or spreading across the substrates. The yellow arrow in A, B, C, and D (i) pointed to cells with thin, elongated filopodia extending from the central body, suggestive of successful attachment, potential migration, or intracellular communication.

Following 28 days of osteogenic induction, pronounced morphological changes were observed, including spreading, elongation, and potential extracellular matrix deposition with calcium nodules, as indicated by the yellow arrow in all images on the right side (red arrows). Meanwhile, image D (iii) showed successful attachment of the hMSCs onto the porous NiTi. The cells demonstrated suggestive progressive attachment, proliferation, and osteogenic differentiation on porous NiTi over time, indicating a strong interaction with the pNiTi substrate, with lamellipodia projecting across the pore structure and filling the pores. These are consistent with extracellular matrix deposition that was quantified using Alizarin Red S Staining in 3.1.3

3.2 Nickel Ion

Figure 6 shows the release of Ni ions in an osteogenic medium and in 0.9% NaCl at pH 6.0 (± 0.2) and 7.0 (± 0.2). Table 1 presents the effects of time, medium, sample group, and their interactions, analyzed using repeated-measures ANOVA. Over 28 days, the pNiTi samples released significantly more Ni ions than the mNiTi samples ($p = 0.02$). Peak Ni ion release occurred on day 14 across all media, followed by a marked decline by day 28. A statistically significant difference was observed in the duration of exposure to the NiTi biomaterials ($p < 0.001$). Multiple pairwise comparisons, adjusted using the Bonferroni correction (as shown in Table 1), indicated significant differences between Day 1 and Day 14, Day 28, and Day 7 and Day 14, and Day 14 and Day 28.

Significant differences were also identified regarding the media utilized ($p < 0.01$). Following Bonferroni correction, significant differences were apparent between DMEM and NaCl at pH 6 and 7 and between NaCl at pH 6 and 7. Notably, mNiTi released significantly higher levels of Ni ions when immersed in 0.9% NaCl at pH 7, whereas pNiTi consistently released elevated Ni ion levels across all media. In DMEM, mNiTi exhibited a steady decline in Ni ion release over a 28-day observation period. Nonetheless, pNiTi demonstrated consistent ion release, with the lowest amount on day 7, followed by a modest increase from day 14 to day 28. The release of Ni ions in DMEM on day 28 by pNiTi was markedly reduced compared to day 1.

Observing the association between Ni ion release and hMSCs activity, no significant relationship was observed, and the hMSCs remained viable throughout the observation period ($r = 0.07$, $p = 0.48$)

Table 1
Multiple Pairwise Comparisons of Effect of Day Exposure, NiTi Group, and Media Used on Nickel Ion Release (µg/L)

Interactions		Mean Difference	95% Confidence Interval for Difference		p-value
			Lower	Upper	
The Effect of Time Exposure (Days)					
Day	Day				
D1	D7	0.002	-0.003	0.008	1.000
	D14	-0.007	-0.013	-0.001	0.018
	D28	0.008	0.003	0.013	< 0.001
D7	D14	-0.009	-0.015	-0.003	< 0.001
	D28	0.006	0.002	0.010	0.001
D14	D28	0.015	0.010	0.020	< 0.001
The Effect of the Group of NiTi Used					
Group	Group				
mNiTi	pNiTi	-0.004	-0.007	-0.001	0.020
The Effect of Media					
Media	Media				
DMEM	NaCl (pH 6)	-0.003	-0.008	0.002	0.380
	NaCl (pH 7)	-0.009	-0.013	-0.004	< 0.001
NaCl (pH 6)	NaCl (pH 7)	-0.006	-0.010	-0.001	0.019
The Effect of Groups Soaking on Different Types of Media					
Group	Media				
mNiTi	DMEM	0.005	0.001	0.009	0.124
	NaCl at pH6	0.008	0.004	0.012	0.189
	NaCl at pH7	0.017	0.013	0.021	< 0.001
pNiTi	DMEM	0.011	0.007	0.015	0.008
	NaCl at pH6	0.014	0.010	0.018	< 0.001
	NaCl at pH 7	0.016	0.012	0.020	< 0.001

4.0 Discussion

This study highlights the promising osteogenic potential of porous nickel-titanium (NiTi) produced using the Metal Injection Molding (MIM) technique, while also investigating nickel ion release over a period of 28 days. The findings emphasize the dual capability of porous NiTi to promote osteoblast-like differentiation in human mesenchymal stem cells (hMSCs) and to evaluate nickel ion release. Significantly, the material exhibited improved cellular proliferation, heightened alkaline phosphatase (ALP) activity, and increased calcium deposition, indicating strong osteogenic properties. Importantly, the release of nickel ions remained well within safe limits, confirming the material's biocompatibility and its potential for clinical applications. These results underscore the advantages of the MIM fabrication technique, which enables the creation of complex porosity that supports essential biological and chemical interactions necessary for successful implant integration.

4.1 Effect of Porous Structure on Osteogenic Behaviour

Implant surface characteristics directly influence cell adhesion. The present study demonstrated that throughout the 28-day incubation period, all biomaterials exhibited higher optical density (OD) values than the negative control group, with porous NiTi implants fabricated via metal injection moulding significantly enhancing osteogenic differentiation of human mesenchymal stem cells compared with machined NiTi and Ti6Al4V control surfaces measured through cell viability, alkaline phosphatase enzyme activities and calcium matrix deposition.

The MTT assay showed a notable increase in optical density (OD) from the first day in porous NiTi, indicating cell viability, metabolic activity, and cellular proliferation. The study indicates that pore shape and size can influence how cells respond, emphasizing the capacity of porous structures to create a microenvironment conducive to cell growth. These findings align with earlier research showing favorable cellular responses to porous NiTi substrates. Hoffman et al and Gotman et al observed greater attachment and proliferation of mesenchymal cells on porous NiTi surfaces [23–24], while Wang et al. found that porous NiTi scaffolds supported cell growth and spreading by enhancing cell–material interactions within the interconnected pore network [25]. Such positive responses may be due to pore design and topography, which increase surface area and facilitate cell attachment, migration, and proliferation by offering multiple anchorage points. For instance, triangular pores with sharp edges are known to aid cell attachment and differentiation [26]. Additionally, porous structures improve nutrient diffusion and metabolic exchange, both essential for cell viability and differentiation. Similar cellular behaviors have been observed in other porous materials, regardless of surface treatments [16]. Conversely, Yu et al. [27] reported decreased cell proliferation and increased apoptosis in porous NiTi compared to machined-surface NiTi and titanium, highlighting the influence of pore size, surface chemistry, and material properties on hMSC behavior. As the incubation period progressed, the OD values for all groups plateaued around day 7 and then declined slightly by day 14. This pattern, consistently observed across all biomaterials, reflects a shift in hMSC behavior. It suggests that as osteogenic differentiation progresses, cellular resources are redirected from proliferation to

differentiation. This competitive interplay between proliferation and differentiation underscores the dynamic nature of osteogenesis, in which one pathway becomes more dominant as the other diminishes [18]. Overall, these results underscore the importance of optimizing pore characteristics to meet both biological and mechanical needs.

To further understand this transition, the study monitored ALP activity and calcium deposition, key markers of early and late osteogenic stages, respectively. These assays are important because mesenchymal cells are multipotent and can differentiate into fibroblasts [19]. Alkaline phosphatase (ALP) activity increased significantly over time, reflecting progression of osteogenic differentiation regardless of substrate type. Although the porous NiTi group demonstrated the highest mean ALP activity at day 14, no statistically significant differences were observed among the biomaterial groups. This finding suggests that while porous NiTi supports osteogenic differentiation, its primary advantage may lie in enhanced matrix maturation and mineralisation rather than in early ALP expression alone. Similar observations have been reported in previous studies, in which differences in mineralisation outcomes were more pronounced than those in early osteogenic markers. The enhanced mineralisation observed in the porous NiTi group is in agreement with previous investigations demonstrating the osteogenic potential of porous NiTi biomaterials. Although ALP activity did not differ significantly among material groups, it increased over time, indicating progression toward osteoblastic differentiation. More importantly, porous NiTi exhibited significantly greater calcium deposition than the control surfaces, suggesting enhanced late-stage osteogenic maturation. Similar findings were reported by Jian et al., who demonstrated increased ALP expression, extracellular matrix formation, and mineralised nodule development on porous NiTi scaffolds. Likewise, Ryan et al. reported that porous metallic biomaterials supported osteoblast differentiation and bone-like matrix formation due to their three-dimensional architecture [28].

The current SEM observations demonstrated progressive attachment, spreading, and colonisation of hMSCs on all tested biomaterials. Cells cultured on porous NiTi exhibited extensive filopodia and lamellipodia extensions, suggesting active interaction with the porous architecture. After 28 days of osteogenic induction, extracellular matrix-like deposits and structures consistent with mineralised nodules were observed within and across the interconnected pore network. These morphological observations are suggestive of osteogenic maturation and support the quantitative findings obtained from ALP and Alizarin Red S assays. However, SEM observations alone cannot definitively confirm osteogenic differentiation and should be interpreted alongside biochemical findings.

4.2 Nickel Ion Release and Biocompatibility

Nickel toxicity remains a major concern in NiTi-based biomaterials. The research investigated nickel ion release under different conditions, including osteogenic medium and saline solutions at pH 6.0 and 7.0, to mimic inflammatory and post-inflammatory environments following implant placement that may affect cellular activity [29–30]. Over 28 days, nickel ion release was tracked, showing a fluctuating pattern across all tested media. Nickel ion concentrations were initially low during the first seven days, likely because few free ions were available on the oxide layer. By day 14, nickel release peaked in saline

solutions for both pNiTi and mNiTi, then declined sharply by day 28. This reduction suggests stabilisation of the material's surface, potentially through the formation of a passive oxide layer that limits further leaching. Interestingly, the osteogenic medium consistently exhibited lower nickel ion release than saline solutions at both pH levels. The medium's buffering capacity and the presence of proteins, calcium, and phosphate ions likely contributed to the formation of a bioactive layer that reduced corrosion and ion leaching. However, nickel ion release was unexpectedly higher in saline at pH 7.0 than at pH 6.0, particularly on day 14. This finding may be attributed to the destabilizing effects of chloride ions, which promote localised corrosion [31]. In contrast, at pH 6.0, self-limiting corrosion mechanisms may dominate, with corrosion products forming a stable protective barrier [21].

Despite these fluctuations, the maximum observed nickel ion concentration ($\sim 0.045 \mu\text{g/L}$) remained well below cytotoxic thresholds [32]. High ALP activity and calcium deposition in the presence of pNiTi confirmed its biocompatibility, indicating that nickel ion release was negligible compared with potential adverse biological effects [33]. The SEM images in Fig. 5d(iii) also support the conclusion that the cells matured and laid down their extracellular matrix. These results align with previous studies highlighting the biocompatibility of NiTi alloys with various surface treatments [34–35]. As bone integration progresses, nickel ion release is expected to decrease further, becoming negligible as the material stabilises within the biological environment [33].

Although pNiTi consistently released slightly more nickel ions than machined NiTi, these levels remained well below cytotoxic thresholds [32] and within safe dietary limits [36]. Nickel, a naturally occurring silvery-white metal, is found in trace amounts in the earth's crust and is excreted by the kidneys. It is present in various environmental sources—including soil, air, metals, and common foods such as chocolate, nuts, grains, and fish—making avoidance nearly impossible [37]. The European Food Safety Authority (2026) recommends a tolerable daily intake of nickel ranging from 2.8 to 13 micrograms per kilogram of body weight (EFSA, 2026) [38].

Porous NiTi produces higher nickel ion release due to its increased surface area from interconnected pores, which exposes more material for ion exchange. Notably, no significant link was found between Ni release and cell viability, indicating biosafety within this setting. Nevertheless, these findings do not eliminate the risk of nickel hypersensitivity or long-term buildup, especially in vivo. Some researchers suggest that surface treatments that may create passive oxide layers or coatings could further reduce ion release without sacrificing biocompatibility [21]. The manufacturing process for porous NiTi plays a pivotal role in minimising impurities and optimising the formation of the NiTi phase (B2), thereby enhancing corrosion resistance. The MIM process directly influences the formation of the complete NiTi phase (B2) and prevents or minimises impurities such as carbon and oxygen, as well as intermetallic phases, and reduces the potential for free Ni ions to leach into the environment [10]. Furthermore, various studies have been conducted to improve its surface to prevent the release of nickel ions, as NiTi has shown significant potential as a bone implant material [27].

Our previous research confirmed that V794 Chinese fibroblast cells are compatible with NiTi extract concentrations up to 200 mg/L, as indicated by USP grade 0 [12]. This is promising because the cells remained intact after exposure, supporting potential future uses. Moreover, polarization tests lasting 30 minutes under open and cyclic conditions show that porous NiTi has excellent corrosion resistance (677 mV) and strong re-passivation ability[33]. The higher breakdown potential, which meets ASTM F2129 standards, indicates adequate corrosion resistance for implants, with a value exceeding 600 mV (U.S. Food and Drug Administration [FDA], 2019) [39]. These findings emphasize the material's self-healing capacity, potentially advancing its resilience [21]

4.3 Study Limitations and Future Directions

The findings of this study suggest that porous NiTi supports osteoconductive processes and maintains nickel-ion release at non-cytotoxic levels. However, the scope of this study was limited to *in vitro* conditions, which do not fully replicate the dynamic biological and mechanical environment of clinical applications. While these results highlight the potential of porous NiTi in implantology, future work should validate these observations under *in vivo* conditions, with particular emphasis on bone integration and long-term material stability.

Exploring surface modifications to further optimize corrosion resistance and enhance osteogenic potential would also be valuable. Additionally, extended incubation periods in simulated physiological environments could provide a deeper understanding of the material's performance over time. Such studies will help bridge the gap between *in vitro* findings and clinical utility.

5.0 Conclusion

This study emphasises the osteogenic capabilities and controlled nickel-ion release of porous NiTi produced through the MIM process. The findings show this material can promote hMSCs' proliferation, differentiation, and mineralization while maintaining nickel ion release below toxic levels. However, several limitations exist. The research was conducted *in vitro*, which does not fully replicate the complex biological, immunological, and mechanical conditions seen in clinical settings. Only one donor-derived hMSC population was tested, limiting insight into individual variability. Osteogenic differentiation was evaluated via ALP activity, mineralization assays, and SEM, without additional gene or protein expression analyses such as RUNX2, osteocalcin, or osteopontin. Additionally, only one porous design and porosity range were examined. Future research should explore various pore geometries, long-term corrosion effects, and *in vivo* osseointegration to better assess the clinical potential of porous NiTi implants.

These findings provide a foundation for developing advanced dental and orthopaedic implants, though further *in vivo* studies are necessary to validate the clinical applicability of porous NiTi under physiological conditions.

Declarations

This manuscript is an original contribution and is not under consideration for publication elsewhere. All authors have reviewed and approved the final version and declare no conflicts of interest. This work was supported by a Research Grant from the Ministry of Science, Technology and Innovation, Malaysia (MOSTI; R&D Grant RD 1019Q1346). The funding body supported the conduct of research, while preparation of this manuscript was completed with institutional support from Universiti Teknologi MARA (UiTM)

Author Contribution

List of Authors and Contributions 1. Nor Wati Nur Atikah Mustafa: Methodology, investigation, formal analysis, writing - original draft2. Muhammad Asif Ahmad Khushaini: Resources (Funding), visualization3. Eshamsul Samsuddin: Conceptualization4. Vivi Noryati Ahmad: Methodology5. Siti Mariam Ab Ghani: Supervision resources6. Zolkapli Eshak: Imaging7. Nurhafizah Kamar Affendi: validation8. Aiemeeza Rajali: Formal analysis9. Muhammad Hussain Ismail: Implant resources10. Rohana Ahmad: Visualization, writing - review & editing

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Figures

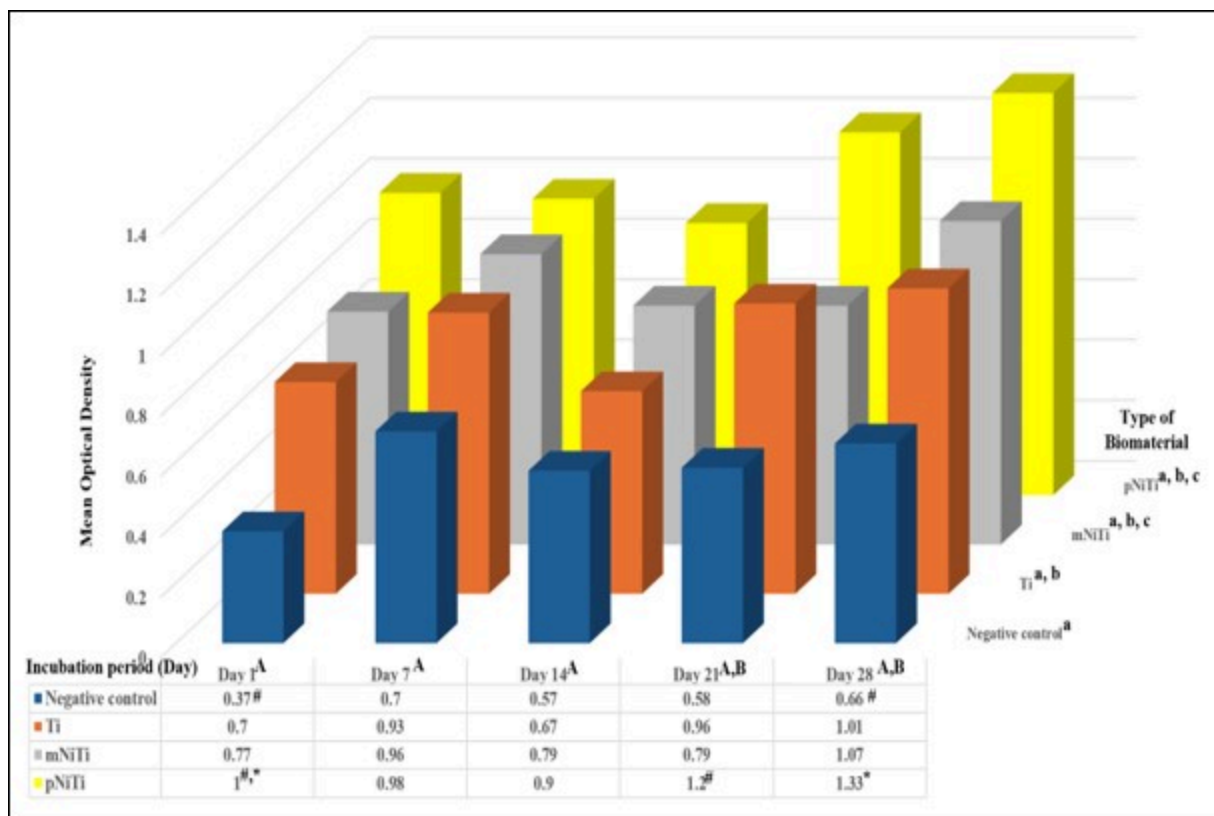


Figure 1

Figure 1. The Effect of Days of Incubation and Type of Biomaterials on Cell Viability. Notes: A repeated-measures ANOVA with within-group and between-group analyses for the time and biomaterials effects, followed by pairwise multiple comparisons with confidence-interval adjustment and Bonferroni correction. A similar capital letter indicates a significant difference within the incubation period, regardless of the biomaterial type. A similar small letter denotes a significant difference between types of biomaterials, and [#] and * denote a significant mean difference in time effect within each group of biomaterials. The significance level is 0.05.

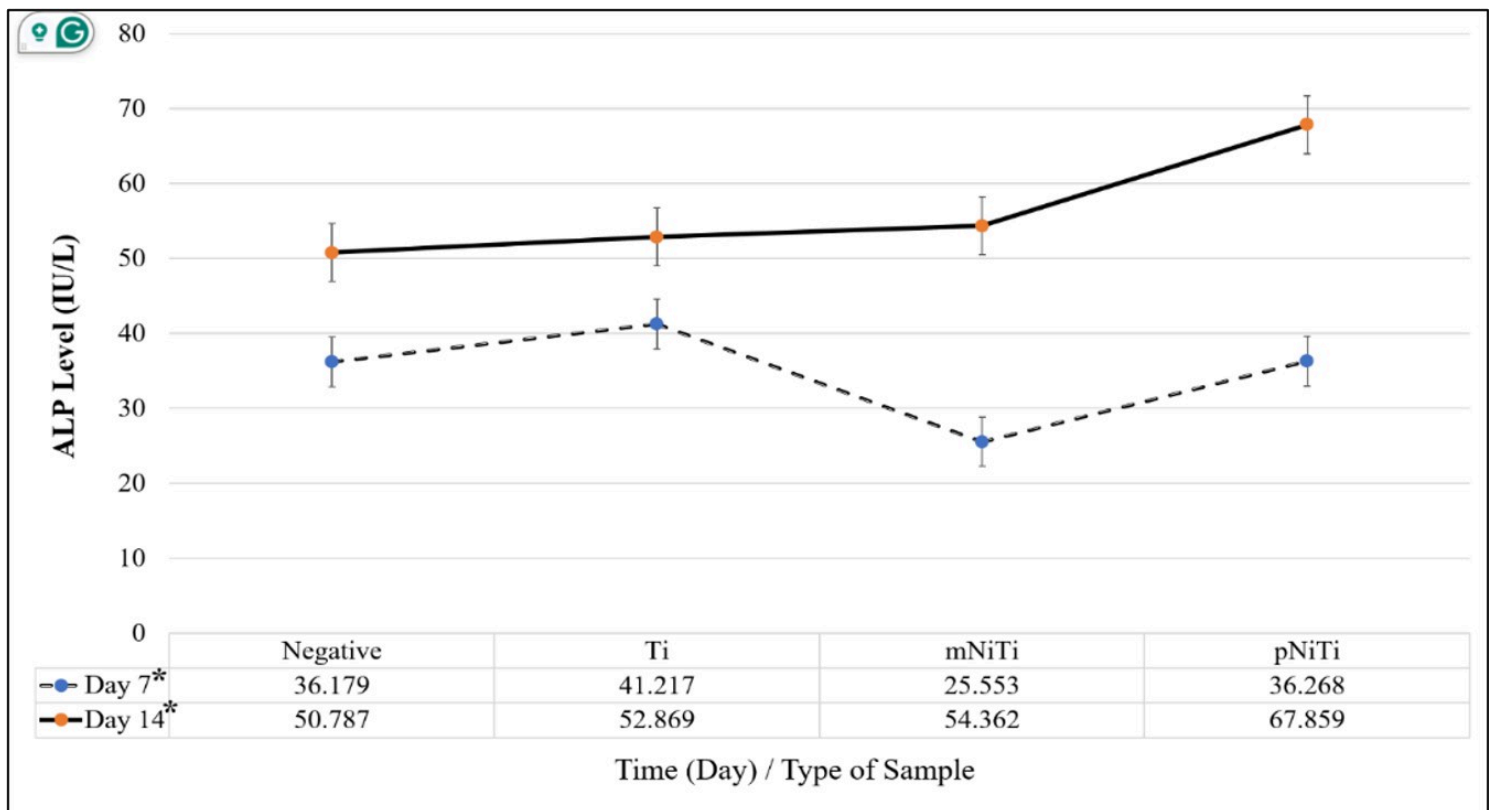


Figure 2

Figure 3. The mean of the ALP level based on the time effect and the type of sample. * Indicates significant difference present, $p < 0.05$

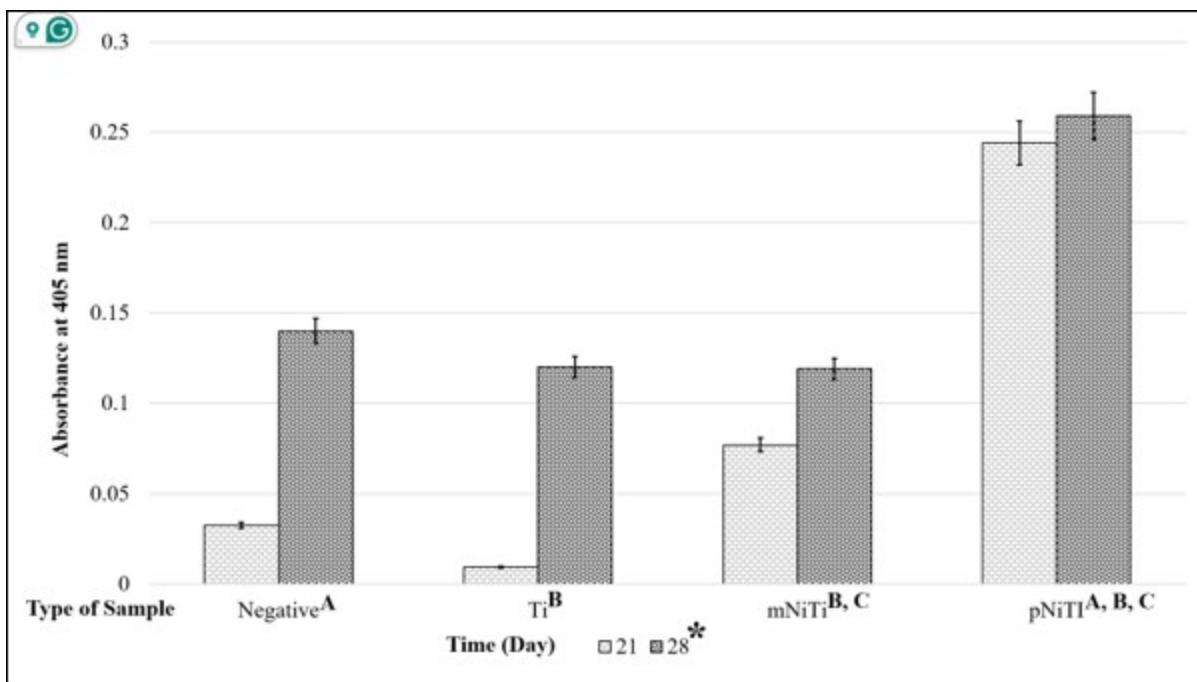


Figure 3

Figure 4. The mean absorbance values at 405 nm on days 21 and 28. * Indicates significant difference present with time, $p < 0.005$. The similar capital letter shows significant differences ($p < 0.001$), while the small letter ($p < 0.05$) between types of samples follows pairwise multiple comparisons with Bonferroni correction.

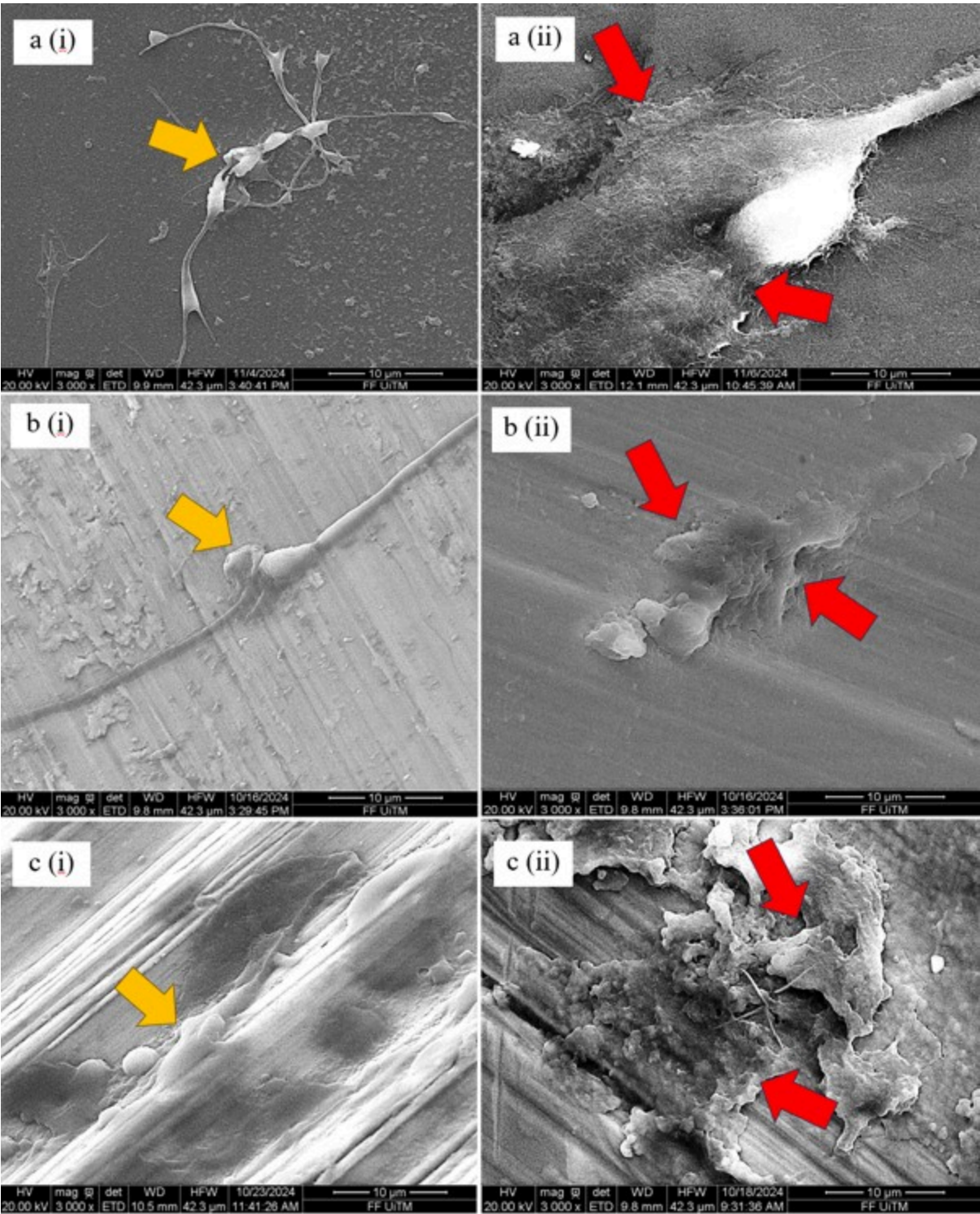


Figure 4

Figure 5. Scanning electron micrographs (SEMs) of human mesenchymal stem cells (hMSCs) cultured in osteogenic media on various biomaterials at days 7 (yellow arrows) and 28 (red arrows) at 3000x magnification. a: Cells on the control surface (glass slide) exhibited (i) on day 7: initial cell adhesion and spreading with an elongated morphology, and (ii) on day 28: increased mineral deposition and significant extracellular matrix formation, indicating osteogenic differentiation. b: Cells on the titanium alloy surface showed (i) day 7: limited attachment and spreading, characterised by reduced cellular interactions, and (ii) day 28: signs of mineralised nodule formation, albeit less pronounced compared to a (ii), c (ii) and porous NiTi in Figure 5 d (iii). c: Cells on a conventional, machined-surface NiTi (mNiTi) demonstrated (i) on day 7: minimal attachment with scattered cellular presence and (ii) on day 28: sparse mineral deposition with restricted osteogenic activity.

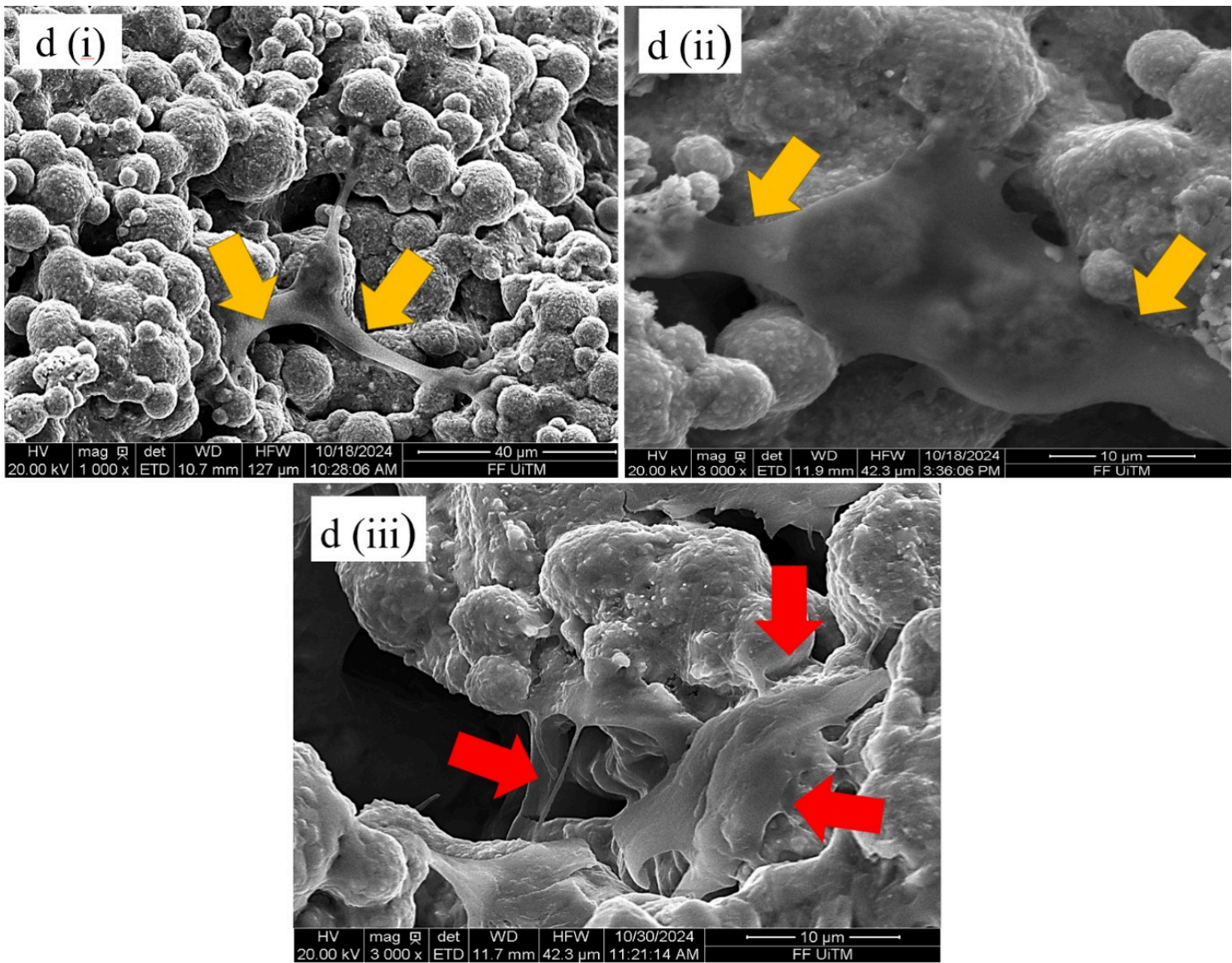


Figure 5

Figure 5 D. Scanning electron micrographs (SEMs) of human mesenchymal stem cells (hMSCs) cultured in osteogenic media on a porous NiTi dental implant at various magnifications and time points. (D i) Day

7 at 1000 x magnification: initial cell adhesion and spreading observed on interconnected porous structures (yellow arrows). (D ii) Day 7 at 3000 x magnification: close-up view of cellular extensions and their interaction with the porous surface (yellow arrows). (D iii) Day 28: Notable deposition of extracellular matrix and the formation of mineralised nodules both within and on the porous surface (red arrows).

(D iii) Day 28: Notable extracellular matrix deposition and mineralised nodules forming within and on the porous surface (red arrows).

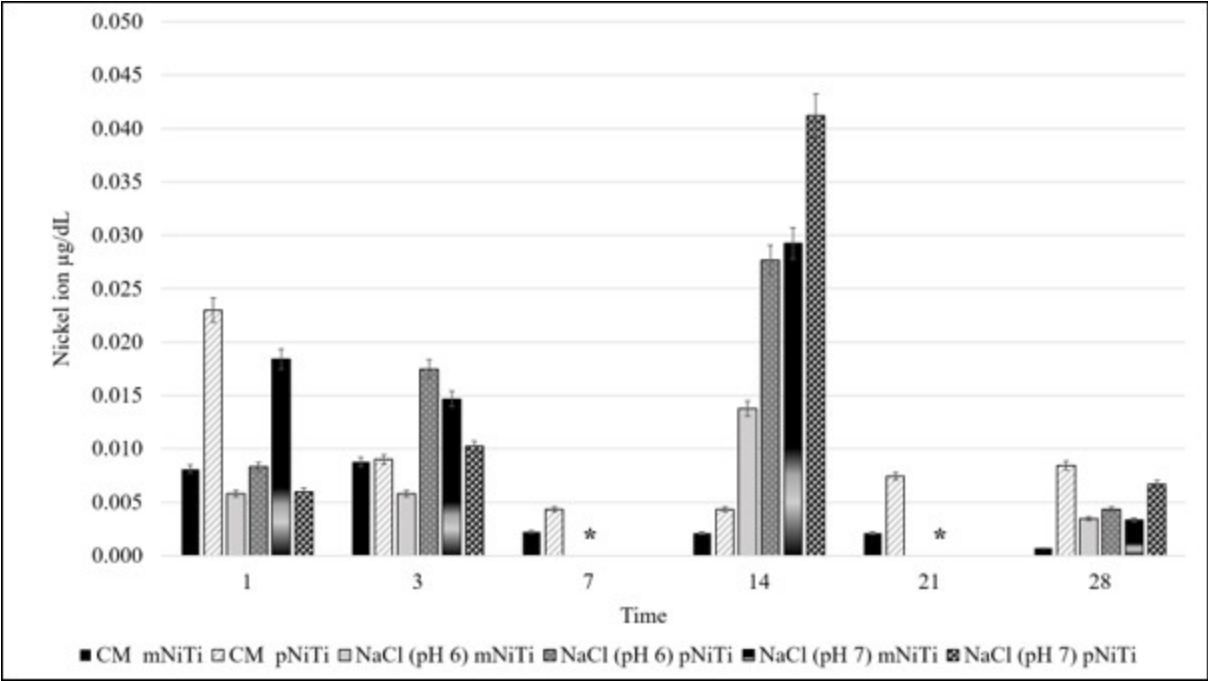


Figure 6

Figure 6. The amount of nickel ion released (µg/L) within a different type of medium by mNiTi and pNiTi. * indicates not perform, p < 0.05, CM: culture medium