Supplementary Information for

Reduced graphene oxide-coated hydroxyapatite composites stimulate spontaneous osteogenic differentiation of human mesenchymal stem cells

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Cytotoxicity assay

The number of viable cells was indirectly quantified using a CCK-8 assay, which contains highly water-soluble tetrazolium salt [WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], reduced to a water-soluble formazan dye by dehydrogenases in cells. Cell viability was found to be directly proportional to the metabolic reaction products obtained in this assay. Briefly, the CCK-8 assay was conducted as follows: The suspension of hMSCs was seeded at a density of 1×10^5 cells ml^{-1} in a 96-well plate and was then cultured in BM at 37 °C under 5% CO_2 until they were grown as monolayer cultures. Cultured cells were treated with increasing concentrations (0 ~ 500 μg ml^{-1}) of HAp microparticles, rGO NPs or rGO-coated HAp composites and were then incubated with WST-8 solution for the last 4 h of the culture period (24 h) at 37 °C in the dark. Since residual rGO NPs can affect the absorbance value at 450 nm, cells exposed to rGO NPs were thoroughly washed with 1 × DPBS prior to incubation with WST-8 solution. Parallel sets of wells containing freshly cultured non-treated cells were regarded as negative (–) controls. The absorbance was determined at 450 nm using an ELISA reader. Relative cell viability was determined as the percentage ratio of the optical density in the medium (containing HAp microparticles or rGO NPs at each concentration) to that in the fresh control medium. The IC_{50}, the concentration (%) inhibiting the growth of cells by 50%, was estimated from relative cell viability profiles.

Cell culture under osteogenic conditions and osteogenesis quantification assays

For osteogenic induction analysis, the suspension of hMSCs was incubated with a colloidal dispersion of HAp microparticles (10 μg ml^{-1}), rGO NPs (10 μg ml^{-1}) or rGO-coated HAp

composites (10 μg ml^{-1}) in osteogenic media (OM) containing 10 mM β-glycerophosphate, (Sigma-Aldrich Co.), 10 nM dexamethansone (Sigma-Aldrich Co.) and 50 μM L-ascorbic acid (Sigma-Aldrich Co.) at 37 °C under 5% CO_2 until they were grown as monolayer cultures. After incubation for 1 to 28 days (or 21 days), the cell proliferation was detected by a CCK-8 assay and osteogenic differentiation was done by an ALP activity assay, ARS staining and Von Kossa staining.

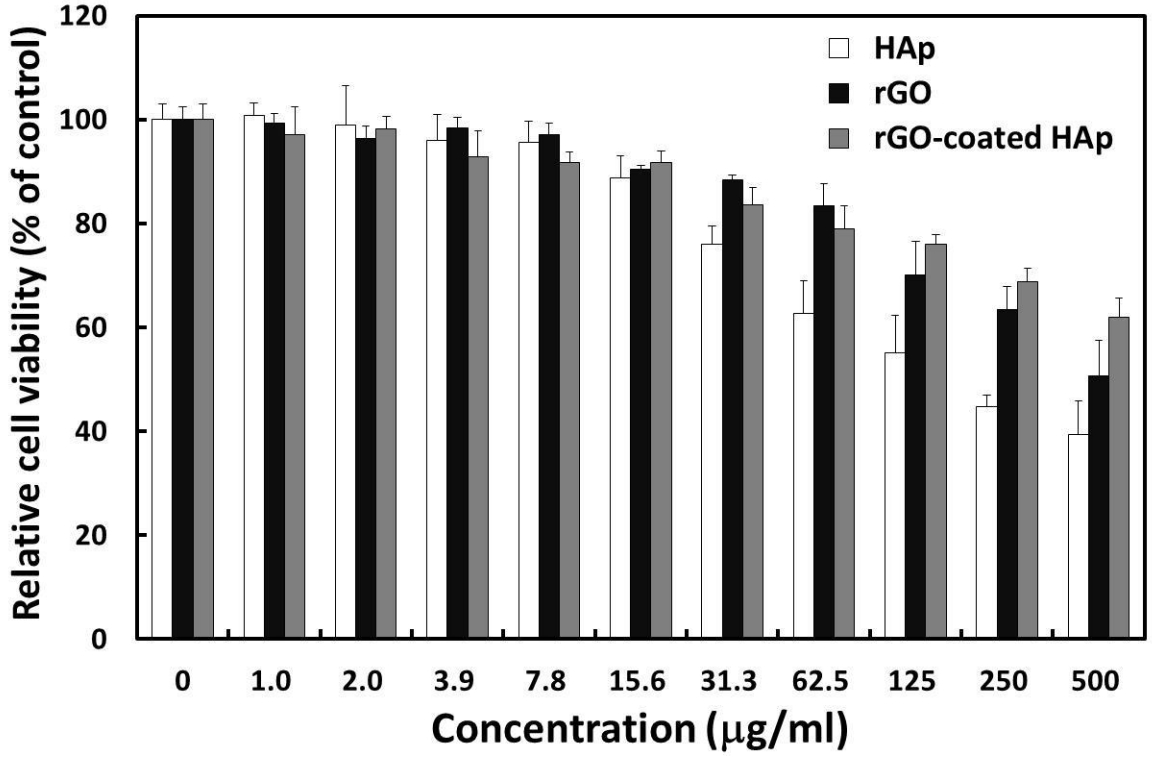


Figure S1. Relative cell viability of hMSCs exposed to HAp microparticles, rGO NPs and rGO-coated HAp composites with increasing concentrations (0 ~ 500 μg ml^{-1}) for 24 h.

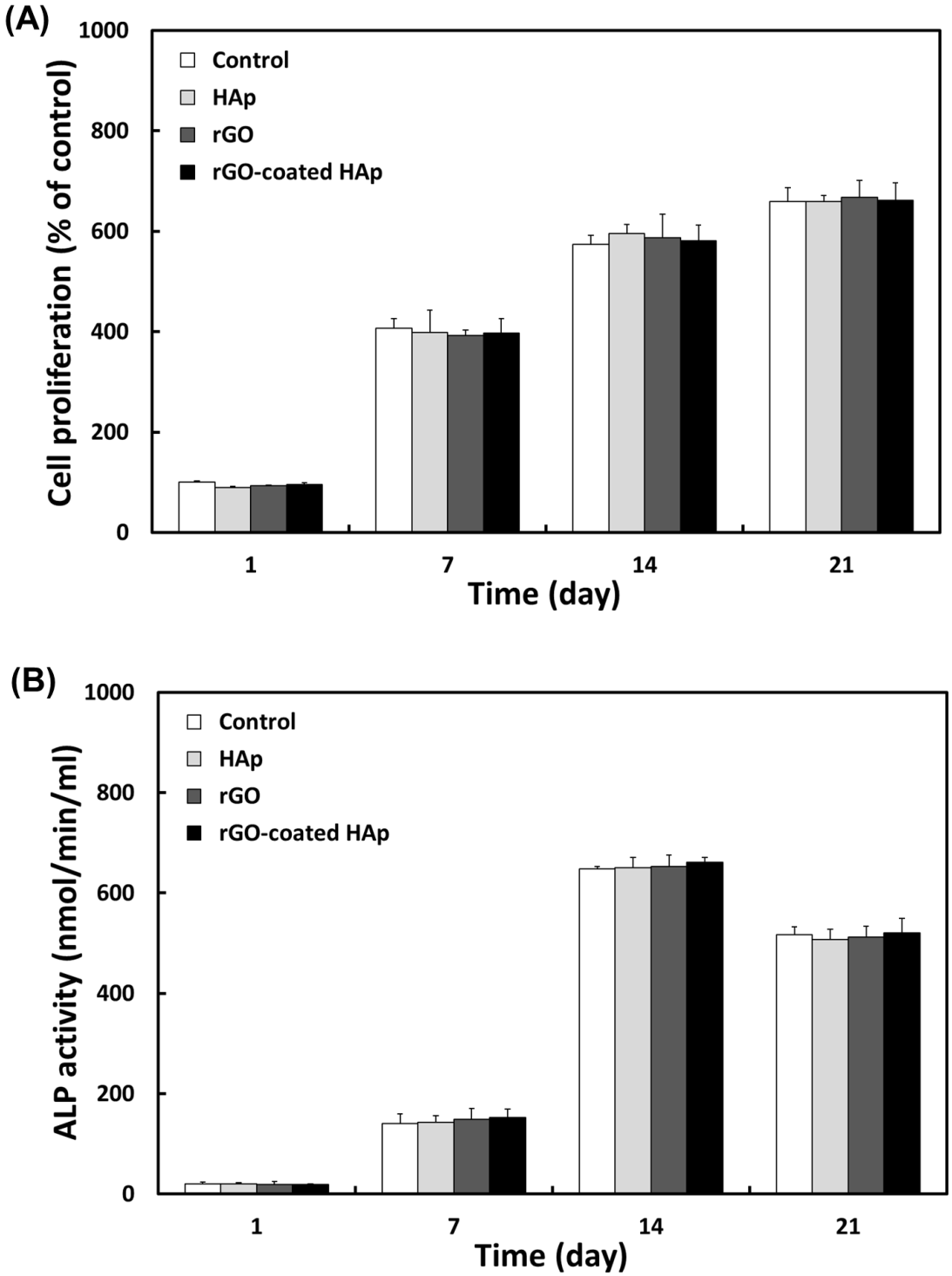


Figure S2. The proliferation and ALP activity of hMSCs incubated with a colloidal dispersion of HAp microparticles, rGO NPs or rGO-coated HAp composites in OM. (A) During the incubation period (up to 21 days), the cell proliferation pattern was almost similar to that of cells cultured in BM regardless of the addition of particles. (B) Cells cultured in OM showed remarkably higher ALP activity from 7 days than cells cultured in BM regardless of the addition of particles. Data were expressed as mean ± SD based on at least duplicate observations from three independent experiments.

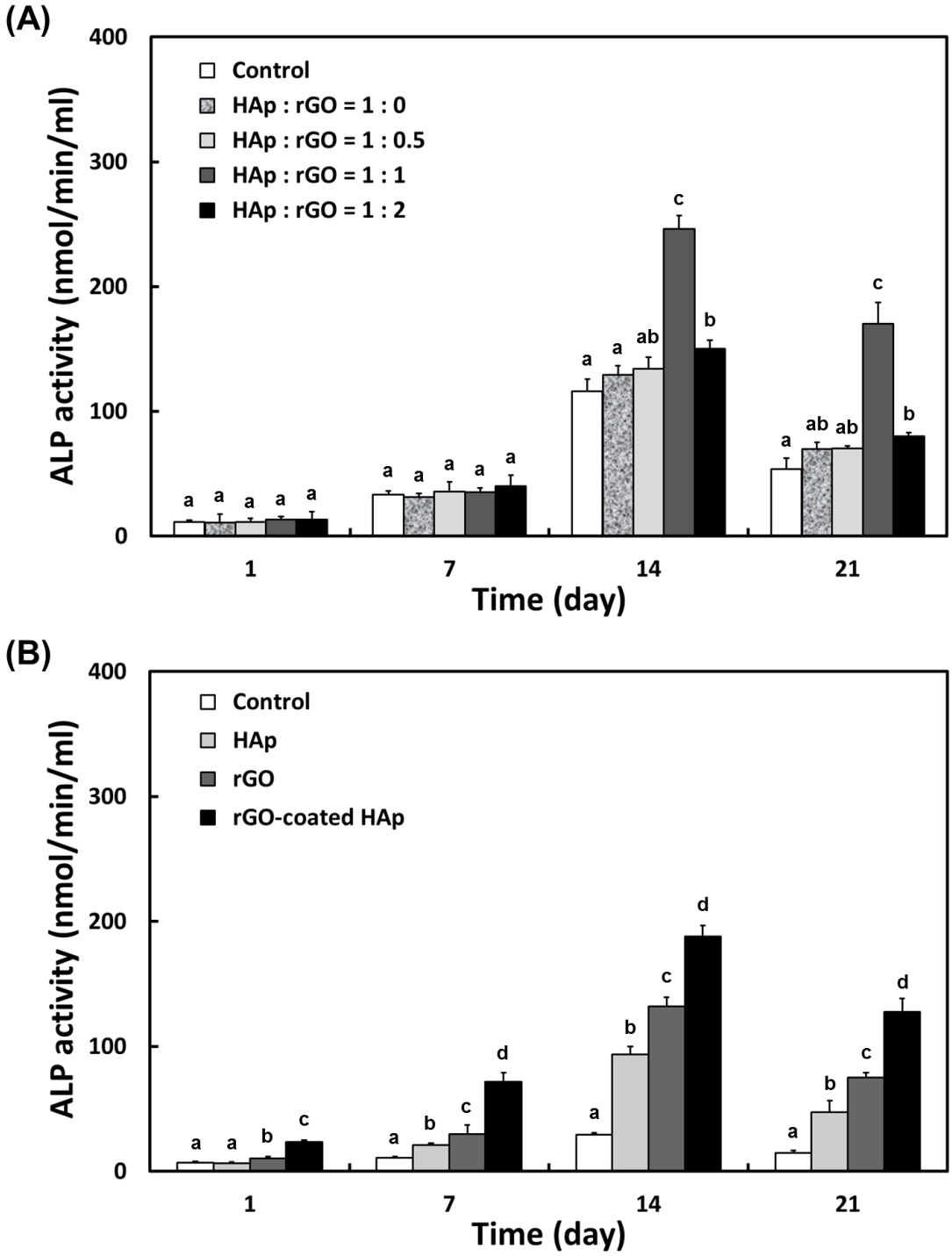
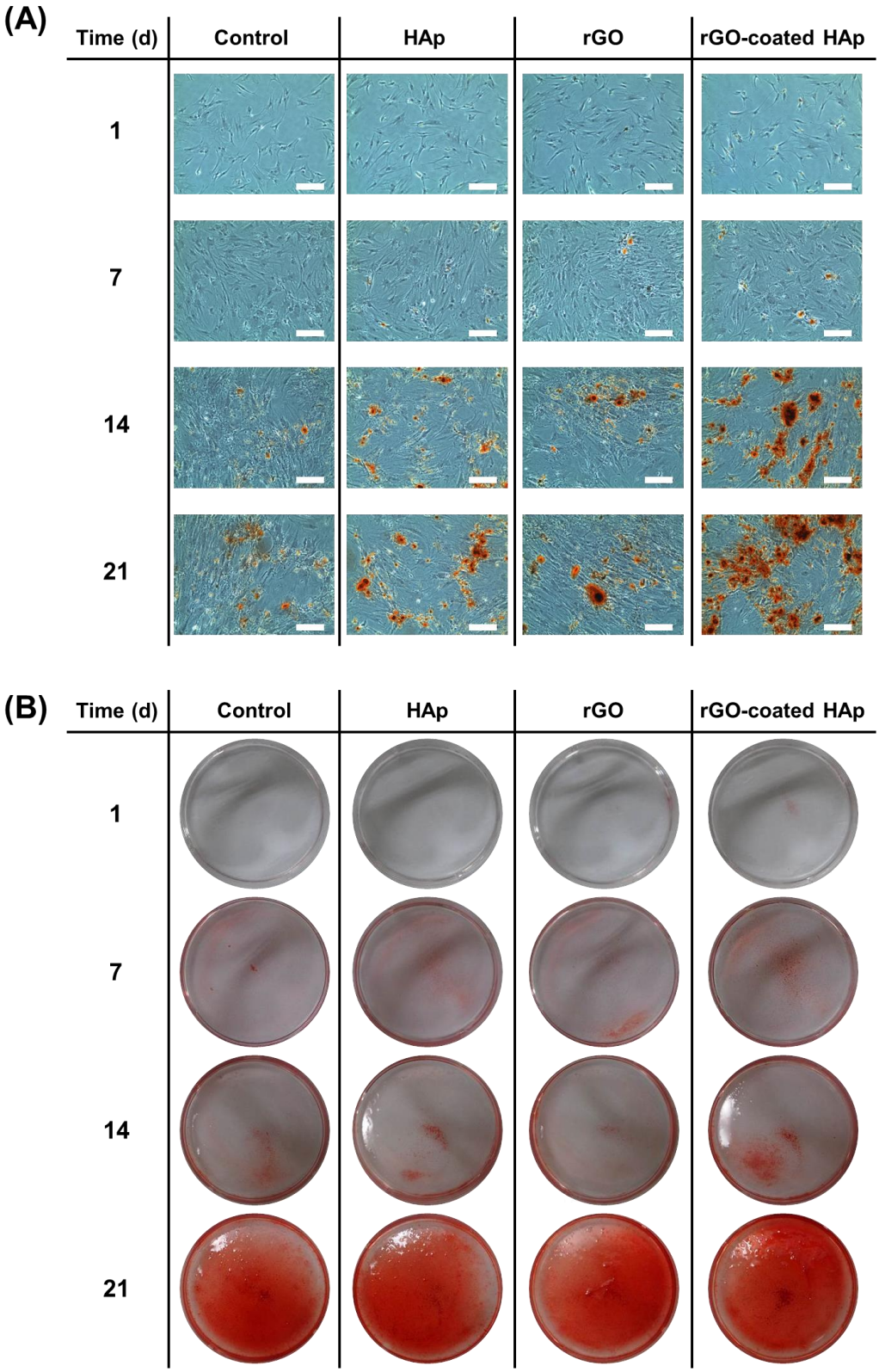


Figure S3. ALP activity of hMSCs (A) incubated with a colloidal dispersion of rGO-coated HAp composites with various ratios of HAp microparticles to rGO NPs in BM and (B) cultured in the 2D incubation system where a colloidal dispersion of HAp microparticles, rGO NPs or rGO-coated HAp composites in BM was treated to as-grown monolayers. Data were expressed as mean ± SD based on at least duplicate observations from three independent experiments. The different letters denote significant differences between the non-treated control and cells incubated with any particles, $p < 0.05$.


(A)
Time (d)
Control
HAp
rGO
rGO-coated HAp
1
7
14
21
(B)
Time (d)
Control
HAp
rGO
rGO-coated HAp
1
7
14
21

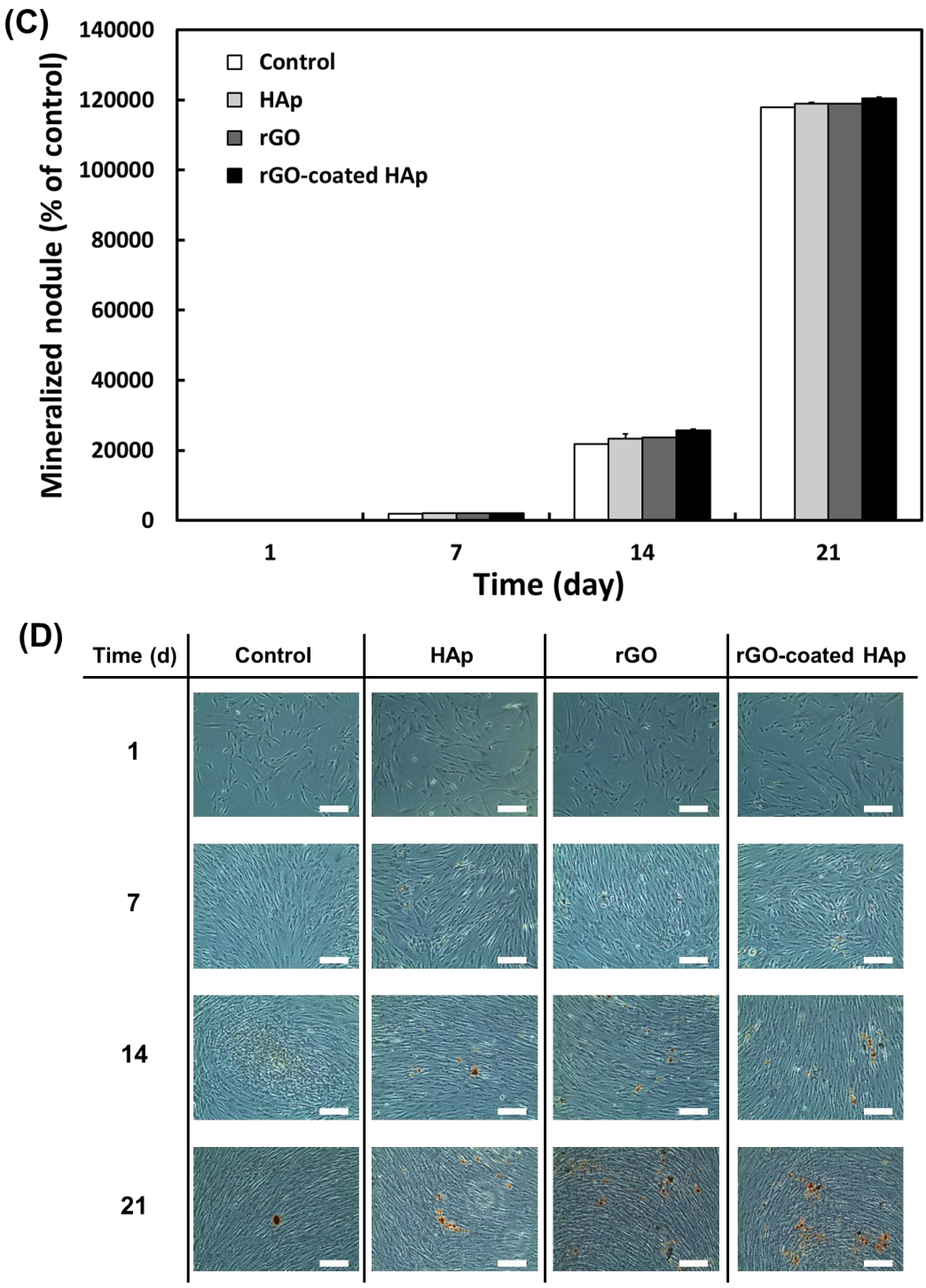


Figure S4. ARS stain in hMSCs incubated with a colloidal dispersion of HAp microparticles, rGO NPs or rGO-coated HAp composites in OM. (A) and (B) There was a notable formation of calcium deposits from 14 to 21 days. In contrast to the ARS staining of cells cultured in BM, it shows a diffuse granular appearance of calcium deposits. (C) rGO-coated HAp composites slightly increased extracellular calcium deposition in cells at 14 and 21 days. (D) The intensity of ARS staining in the 2D incubation system was much lower than that in the 3D incubation system. Data were expressed as mean ± SD based on at least duplicate observations from three independent experiments. All photographs shown in this figure are representative of six independent experiments with similar results. Scale bars are 200 μm in (A) and (D).

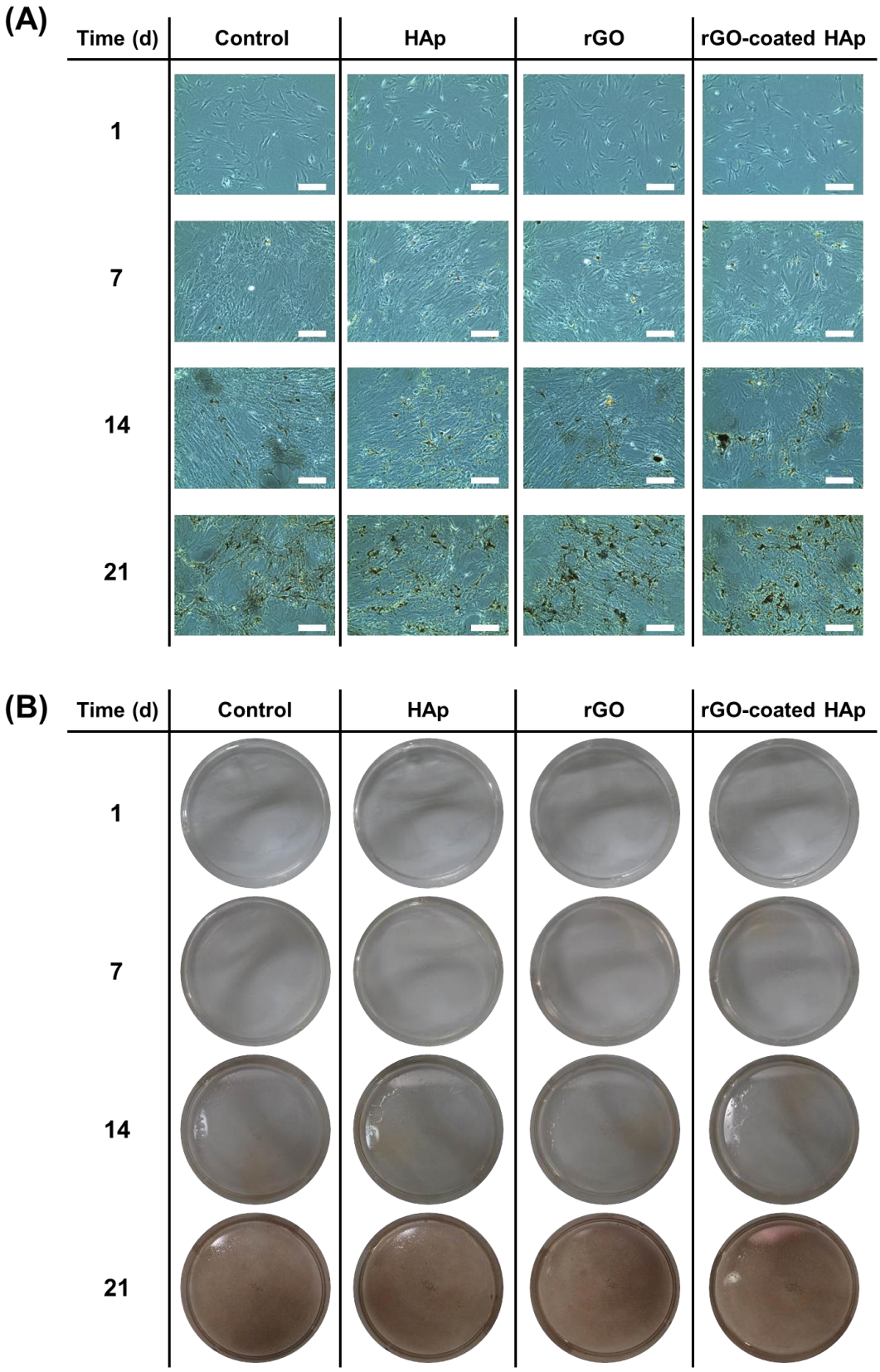


Figure S5. The image of von Kossa stain in hMSCs incubated with a colloidal dispersion of HAp microparticles, rGO NPs or rGO-coated HAp composites in OM. Dark brown mineralized nodules (A) and crystal formation (B) were observed even at 21 days irrespective of the addition of particles [scale bars = 200 μm in (A)]. All photographs shown in this figure are representative of six independent experiments with similar results.