

N-acetylcysteine (NAC) differentially affects arterial medial calcification and bone formation: The role of L-cysteine and hydrogen sulphide

Lucie E. Bourne¹ | Jessal J. Patel¹ | Bethan K. Davies¹ | Ellen Neven² |
Anja Verhulst² | Patrick C. D'Haese² | Caroline P. D. Wheeler-Jones¹ |
Isabel R. Orriss¹ 

¹Department of Comparative Biomedical Sciences, Royal Veterinary College, London, UK

²Department of Biomedical Sciences, Laboratory of Pathophysiology, University of Antwerp, Antwerp, Belgium

Correspondence

Isabel R. Orriss, Department of Comparative Biomedical Sciences, Royal Veterinary College, Royal College St, London, NW1 0TU, UK.

Email: iorriss@rvc.ac.uk

Funding information

The Rosetrees Trust, Grant/Award Number: M744; British Heart Foundation, Grant/Award Number: PG/15/13/31296

Abstract

Arterial medial calcification (AMC) is the deposition of calcium phosphate in the arteries. AMC is widely thought to share similarities with physiological bone formation; however, emerging evidence suggests several key differences between these processes. N-acetylcysteine (NAC) displays antioxidant properties and can generate hydrogen sulphide (H₂S) and glutathione (GSH) from its deacetylation to L-cysteine. This study found that NAC exerts divergent effects in vitro, increasing osteoblast differentiation and bone formation by up to 5.5-fold but reducing vascular smooth muscle cell (VSMC) calcification and cell death by up to 80%. In vivo, NAC reduced AMC in a site-specific manner by 25% but had no effect on the bone. The actions of L-cysteine and H₂S mimicked those of NAC; however, the effects of H₂S were much less efficacious than NAC and L-cysteine. Pharmacological inhibition of H₂S-generating enzymes did not alter the actions of NAC or L-cysteine; endogenous production of H₂S was also unaffected. In contrast, NAC and L-cysteine increased GSH levels in calcifying VSMCs and osteoblasts by up to 3-fold. This suggests that the beneficial actions of NAC are likely to be mediated via the breakdown of L-cysteine and the subsequent GSH generation. Together, these data show that while the molecular mechanisms driving the actions of NAC appear similar, the downstream effects on cell function differ significantly between osteoblasts and calcifying VSMCs. The ability of NAC to exert these differential actions further supports the notion that there are differences between the development of pathological AMC and physiological bone formation. NAC could represent a therapeutic option for treating AMC without exerting negative effects on bone.

KEYWORDS

bone formation, hydrogen sulphide, L-cysteine, N-acetylcysteine, vascular calcification

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1 | INTRODUCTION

Blood vessel calcification is a common consequence of ageing, atherosclerosis, diabetes, and chronic kidney disease (CKD). It is the pathological deposition of calcium phosphate mineral, often as hydroxyapatite, in the arteries and heart valves. Arterial medial calcification (AMC) refers to the calcification, which occurs within the tunica media of blood vessels and is characterised by increased vessel stiffness and reduced blood flow (Young et al., 1993). Traditionally, AMC was viewed as a passive process caused by high serum levels of calcium and phosphate. However, it is now accepted that AMC is a complex cell-mediated process that may share some outward similarities to physiological bone formation. Whilst many cells can contribute to AMC, vascular smooth muscle cells (VSMCs) are thought to be the major cell type driving this process (Narisawa et al., 2007; Zhu et al., 2011). Many studies have shown that within a calcifying environment (high phosphate and/or calcium), VSMCs can undergo phenotypic changes to assume some characteristics usually associated with bone-forming osteoblasts (Jono et al., 2000; Lomashvili et al., 2006; Shanahan et al., 2011; Zhu et al., 2011). However, while VSMCs do transdifferentiate to some extent under these conditions they should not be considered equivalent to mature osteoblasts (Alves et al., 2014; Patel et al., 2019a).

To develop effective therapies for AMC that do not exert negative actions on the skeleton, it is important to understand both the similarities and differences between AMC and bone formation (Bourne et al., 2021). Reactive oxygen species (ROS) are signalling molecules known to have both beneficial and deleterious effects on disease initiation and progression (Zuo et al., 2015). A number of studies have reported opposing effects of ROS and antioxidants on AMC and bone formation. Increased ROS levels have been implicated in driving the development of AMC (Byon et al., 2008; Muteliefu et al., 2009), while VSMC calcification is reduced by antioxidants (e.g., resveratrol, quercetin, and curcumin) (Cui et al., 2017; Hou et al., 2016; Zhang et al., 2016). In contrast, high levels of ROS inhibit osteoblast differentiation and reduce bone formation (Fatokun et al., 2008; Kim et al., 2010; Mody et al., 2001) whereas antioxidants can stimulate these processes (Dai et al., 2007; Orriss et al., 2016; Pang et al., 2018). Thus, understanding the mechanisms underpinning these differential effects could be of significance for future drug development (Bourne et al., 2021).

N-acetylcysteine (NAC), the acetylated form of the amino acid L-cysteine, has a range of clinical uses (see Dodd et al., 2008; Lasram et al., 2015). NAC displays direct and indirect antioxidant properties as well as anti-inflammatory and antiapoptotic effects (Lasram et al., 2015). It also directly scavenges a number of ROS, including hydrogen peroxide (H₂O₂) and nitrogen dioxide. However, its status as an antioxidant is thought to stem from its role as a precursor to L-cysteine, an amino acid that is the rate-limiting substrate in the formation of glutathione (GSH), a key intracellular antioxidant (Dodd et al., 2008; Lasram et al., 2015). The generation of GSH from L-cysteine requires the enzymes glutamate cysteine ligase (GCL) and glutathione synthetase (GSS). Importantly, due to its thiol-containing

moiety, L-cysteine can also be used in the generation of hydrogen sulphide (H₂S). H₂S is a gaseous signalling molecule with a number of cardioprotective effects (Stein & Bailey, 2013) that is primarily generated via the actions of cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CSE). Expression of CBS and CSE has been reported in the vasculature and bone (Castelbanco et al., 2020; Donovan et al., 2017; Robert et al., 2005; Zavadzki et al., 2011).

NAC has been widely used experimentally in the context of ameliorating the negative actions of ROS on cells (Zafarullah et al., 2003). For example, NAC has been shown to reduce hypoxia or salusin- β -induced VSMC calcification (Balogh et al., 2019; Sun et al., 2019). Nonetheless, the direct actions of NAC on AMC remain to be fully described. In bone, previous work has suggested that NAC exerts beneficial effects by promoting osteoblast differentiation and bone formation *in vitro* and *in vivo* (Jun et al., 2008; Romagnoli et al., 2013; Yamad et al., 2013).

Despite the reported similarities between AMC and bone formation the two processes are usually investigated in isolation and studies which directly compare them are limited (Mody et al., 2001; Patel et al., 2018; Patel et al., 2019a). The aims of this investigation were to: (1) determine the effects of NAC on AMC and bone formation in parallel; (2) establish the mechanisms underpinning any beneficial actions; and (3) identify fundamental differences in the processes of bone formation and AMC, which could potentially be exploited therapeutically.

2 | METHODS

2.1 | Reagents

All tissue culture reagents were purchased from Thermo Fisher Scientific; unless mentioned, all chemicals were obtained from Sigma Aldrich. Primary antibodies were purchased from Abcam UK (TNAP, OPN, β -actin, α -SMA, SM22 α , CBS, and CSE) or Cell Signalling Technology (Runx2). Secondary antibodies were from Jackson ImmunoResearch Europe.

2.2 | Animals

All animal procedures complied with the UK Animals (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Research Ethics Committee. All animals were group-housed under standard conditions with free access to food and water.

2.3 | Osteoblast bone formation assay

Osteoblasts were isolated from the calvariae of 3–5 day old C57Bl/6J mice by trypsin/collagenase digestion as previously described (Perpetuo et al., 2019; Taylor et al., 2014). Cells were expanded in Alpha Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS),

2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (mixture abbreviated to α MEM). Following plating in 6-well trays at 10^5 cells/well, osteoblasts were cultured for up to 21 days in α MEM supplemented with 1 mM β -glycerophosphate, 1 mM sodium phosphate dibasic and 50 µg/ml ascorbic acid, with half medium changes every 3 days. Time points in osteoblast cultures were: proliferating (Day 4), differentiating (Day 7), mature (Day 14), and mature, bone forming (Day 21). All experiments were performed on cells that were isolated, expanded and plated; the cells were not passaged at any stage. Osteoblasts were treated with NAC (0.5–2.5 mM), L-cysteine (0.5–2.5 mM), NaHS (50–300 µM), or GSH (reduced form, 1–100 µM) for the entire culture. For some experiments, the pharmacological inhibitors, DL-propargylglycine (PAG, 100 µM) or aminooxyacetic acid (AOAA, 100 µM) were used alone or in conjunction with L-cysteine or NAC (2.5 mM).

Experiments were terminated by fixing the cells in 2.5% glutaraldehyde for 5 min. Plates were imaged at 800 dpi using a flat-bed scanner and the total area of bone nodules was quantified by image analysis, as described previously (Perpetuo et al., 2019; Taylor et al., 2014).

2.4 | VSMC calcification assay

Human VSMCs were isolated from umbilical arteries via explant culture and mouse VSMCs were obtained from isolated aortas as previously described (Patel et al., 2016; Patel et al., 2019a). Cells were expanded in Dulbecco's MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin (mixture abbreviated to DMEM). All experiments were performed on human cells between Passage 3–5. Mouse VSMCs were isolated, expanded and plated. All procedures involving human tissue complied with the UK Human Tissue Act (2004). Human umbilical cord collection (obtained with informed written consent) and the use of arterial smooth muscle cells conformed to the principles outlined in the Declaration of Helsinki and is approved by the NHS Health Research Authority East of England-Cambridge South Research Ethics Committee (REC reference 16/EE/0396).

To induce calcification, VSMCs were plated in 24-well trays at 2.5×10^4 cells/well and cultured for up to 7 days in the same conditions as osteoblasts (i.e., α MEM supplemented with 1 mM β -glycerophosphate, 1 mM sodium phosphate dibasic, and 50 µg/ml ascorbic acid), with half medium changes every 3 days. Time points in VSMC cultures were: early calcification (Day 4) and widespread calcification (Day 7). VSMCs were exposed to NAC, L-cysteine, NaHS, or GSH and where appropriate PAG or AOAA, for the entire culture (concentrations as above).

2.5 | Determination of VSMC calcification

Calcifying VSMCs were washed with phosphate-buffered saline (PBS) and incubated with 0.6 M HCl at room temperature for 24 h. Calcium content was measured colorimetrically by stable interaction with

o-cresolphthalein using a commercially available kit and corrected for total protein concentration using the Bradford assay (both Sigma-Aldrich). Calcium deposition was visualised by alizarin red staining of VSMC cell layers as previously described (Patel et al., 2018; Patel et al., 2019a).

2.6 | Cell viability assay

VSMCs and osteoblasts were cultured for less than or equal to 7 and 21 days, respectively. Cell viability was determined using the CytoTox 96[®] colorimetric cytotoxicity assay (Promega UK), as described previously (Patel et al., 2018). Cell supernatants were collected to determine medium lactate dehydrogenase (LDH) levels (cell viability). To establish total cellular LDH levels, cells were lysed with 1% Triton X-100 in water for 1 h. The LDH content of the supernatants and cell lysates were measured colorimetrically (495 nm) as per the manufacturer's instructions. Cell viability (% dead cells) was calculated by expressing medium LDH as a percentage of the total cellular LDH.

2.7 | Determination of alkaline phosphatase (TNAP) activity

VSMCs and osteoblasts were cultured for less than or equal to 7 and 21 days, respectively. TNAP activity was measured in cell lysates using a colorimetric assay (Anaspec), as previously described (Perpetuo et al., 2019) and normalised to cell protein using the Bradford assay.

2.8 | Total RNA extraction and DNase treatment

Osteoblasts were cultured for 4, 7, or 14 days before total RNA was extracted using Qiazol[®] reagent (Qiagen Ltd) according to the manufacturer's instructions. VSMCs were cultured for 4 or 7 days before RNA collection. Extracted RNA was treated with RNase-free DNase I (35 U/ml) for 30 min at 37°C. The reaction was terminated by heat inactivation at 65°C for 10 min. Total RNA was quantified spectrophotometrically by measuring absorbance at 260 nm (Nanodrop 1, Thermo Fisher UK).

2.9 | Quantitative real-time polymerase chain reaction (qRT-PCR)

Osteoblast and VSMC RNA (50 ng) was transcribed and amplified using the qPCRBIO SyGreen one-step quantitative real-time polymerase chain reaction (qRT-PCR) Kit (PCR Biosystems). qRT-PCR was performed according to the manufacturer's instructions with initial cDNA synthesis (45°C, 10 min) and reverse transcriptase inactivation (95°C, 2 min) followed by 40 cycles of denaturation (95°C, 5 s) and detection (60°C, 30 s). All reactions were carried out in triplicate using RNAs derived from 4 to 5 different cultures. Data were

analysed using the $\Delta\Delta\text{Ct}$ method (Livak & Schmittgen, 2001). Primers were obtained from Qiagen Ltd.

2.10 | Western blot analysis

Protein was extracted from osteoblasts and VSMCs at 4, 7, and 14 days. Cells were lysed in ice-cold radio immunoprecipitation (RIPA) buffer, sonicated for 5 min and homogenates stored at -80°C for at least 30 min before use. Lysate protein concentrations were determined using the Bradford assay. Protein samples were denatured by incubating at 100°C for 5 min in the presence of 5 \times reducing sample buffer before loading (20 $\mu\text{g}/\text{lane}$) onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) gels and transferred onto a polyvinylidene difluoride membrane (Amersham) by the use of a wet tank blotter (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated with β -actin (1:5000), Runx2 (1:1000), TNAP (1:20,000), OPN (1:1000), α -SMA (1:500), SM22- α (1:500), CBS (1:1000), or CSE (1:1000) antibodies overnight at 4°C . After washing, blots were incubated in horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. A peroxidase detection system (ImmobilonTM Western, Merck-Millipore) and ChemiDocTM XRS + system (Bio-Rad) were used for the visualisation of immunoreactivity. Quantification of blots was performed using ImageJ software with band densities normalised to the β -actin loading control to show the relative change in protein expression.

2.11 | In vivo study to determine the effects of NAC on AMC and bone mass

This investigation used 22 male 8-week-old Wistar rats, with each being approximately 350 g at the start of the study (Charles River). Animals were housed two per cage and exposed to a 12 h light/dark cycle with free access to food and water. AMC was induced in all animals by feeding a warfarin (3.2 g/kg) and vitamin K1 (1.5 g/kg) enriched diet (S8893-E080, SSNIF) for 10 weeks, as previously described (Neven et al., 2015). Rats were randomly allocated to the control or treatment groups and dosed daily with vehicle or NAC (oral gavage, 300 mg/kg/day, Sigma-Aldrich) for the duration of the study. No adverse effects associated with the altered diet or NAC treatment were observed in either experimental group. However, three rats were euthanised during the study for reasons unrelated to the protocol; these animals were excluded from the data analysis (1 control and 2 test). Upon study termination, animals were euthanised and tissues collected and processed for analysis.

2.12 | Microcomputed X-ray tomographic (μCT) analysis of AMC and bone parameters

The tibiae, femora, and whole aorta were isolated from control and NAC-treated rats at the end of the in vivo study. Tissues were fixed

in 10% neutral-buffered formalin (NBF) for 24 h, PBS washed and then stored in 70% ethanol. The microcomputed tomographic (μCT) scanner (SkyScan 1172) was set at 50 kv and 200 μA using a 0.5 mm Al filter and a resolution of 7 μm . The aortas exceeded the maximum sample size for the μCT machine and were cut at the halfway point between the aortic arch and bifurcation. The resulting two sections were (1) top, comprising the aortic arch to the thoracic aorta and, (2) bottom, comprising the abdominal aorta to the bifurcation.

All μCT scans were reconstructed, analysed, and visualised using established protocols (Hajjawi et al., 2014; Orriss et al., 2017) and the SkyScan NRecon, CTAn, and CTVol software. Briefly, morphometric analysis of trabecular and cortical bone parameters was performed on the tibial and femoral metaphysis and diaphysis. The appearance of the first trabecular bridge connecting the four primary spongiosa bone "islands" was used as a reference point, with an offset of 1.5 mm and 5 mm for metaphyseal trabecular and cortical bone, respectively. The length of the bone analysed was 2 mm in both regions. Bone mineral density (BMD) was calibrated and calculated using hydroxyapatite phantoms with a known density. To ensure that all AMC was included in the analysis, quantification was performed on both sections of the aorta. Scanning and analysis of all tissues were performed blind.

2.13 | Detection of H_2S levels in vitro

VSMCs and osteoblasts were cultured in 35 mm glass-bottomed cell culture dishes for 4 and 7 days, respectively. The fluorescent probe sulphide fluor-7 acetoxymethyl ester ($\text{SF}_7\text{-AM}$) was used to detect the presence of H_2S in cells as described by Lin et al. (2013). Briefly, cells were incubated with $\text{SF}_7\text{-AM}$ (5 μM) for 30 min at 37°C . Baseline images were taken before cells were treated with NAC or L-cysteine (2.5 mM); the addition of NaHS (300 μM) was used as a positive control. Images were taken from pre-determined locations every 5 min for 30 min using a Leica DMI6000 motorised microscope with a heated stage. All camera settings remained the same throughout.

2.14 | Measurement of glutathione (GSH) levels

Total glutathione (GSH) was measured in osteoblasts and VSMCs cultured for less than or equal to 14 and 7 days, respectively. GSH levels were assessed using a colorimetric assay based on the method by Rahman et al. (2006). Cell monolayers were collected in PBS and spun at 1000 g at 4°C for 5 min. The pellet was resuspended in ice-cold extraction buffer (0.1% Triton-X, 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM ethylenediaminetetraacetic acid, pH7.5) and homogenised. Lysed cells were sonicated for 3 min with vortexing every 30 s before being centrifuged at 3000 g for 5 min at 4°C . Total GSH in collected supernatants was measured following addition of 5,5-dithio-bis (2-nitrobenzoic acid) (0.66 mg/ml), glutathione reductase (3 U/ml) and NADPH (0.66 mg/ml) using a microplate reader (412 nm). Absorbance values were compared to a standard curve of known GSH concentrations and normalised for total protein using the Bradford assay.

2.15 | Statistics

Data were analysed using GraphPad Prism 8 software. All data are presented as bar graphs with points to show the values for individual experiments (in vitro) or animals (in vivo). In vitro results show data from 4 to 6 individual experiments; each experiment was performed using cells isolated from different animals (osteoblasts) or umbilical cords (VSMCs). Within each experiment, each group contained 3–6 technical replicates. To account for the inherent variation of using primary cells isolated from different animals/cords, all in vitro data were analysed using randomised block ANOVA followed by Fisher's LSD post hoc test as described by Festing (Festing, 2001). In vivo data are from 9 to 10 animals; results were analysed using an unpaired student's *t*-test.

3 | RESULTS

3.1 | NAC inhibits VSMC calcification

Culture with NAC dose-dependently inhibited VSMC calcification by 65%, 80%, and 73% at 0.5 mM, 1 mM and 2.5 mM, respectively ($p < 0.05$, Figure 1a). A reduction in calcification of up to 80% was also observed in mouse VSMCs ($p < 0.001$, Figure S1a). Compared

with calcifying medium alone, NAC treatment (≥ 1 mM) reduced the percentage of cell death by up to 40% and 35% at Day 4 and Day 7, respectively ($p < 0.05$, Figure 1b). NAC had no effect on VSMC TNAP activity at any stage (Figure 1c). Representative phase-contrast microscopy images of alizarin red-stained VSMCs show the decreased calcification in NAC-treated cultures (Figure 1d).

3.2 | NAC promotes bone formation by osteoblasts

Untreated osteoblasts displayed only limited mineralised bone nodule formation after 14 days of culture (≤ 4 mm²). Conversely, NAC treatment increased the level of bone formation by 2.5-fold and 5.5-fold at 1 mM and 2.5 mM, respectively ($p < 0.05$, Figure 2a). This stimulatory effect was smaller by Day 21 with NAC only increasing bone formation by 1.6-fold, 2.2-fold, and 3-fold at 0.5 mM, 1 mM, and 2.5 mM, respectively ($p < 0.05$, Figure 2a). NAC (1–2.5 mM) increased osteoblast TNAP activity by up to 2-fold at Day 14; a trend to increased activity was also seen at Day 21 (Figure 2b). NAC had no effect on osteoblast viability at any stage (Figure 2c). Representative whole-well scans and phase-contrast images show the increased bone formation at both Day 14 and Day 21 in NAC-treated osteoblasts (Figure 2d).

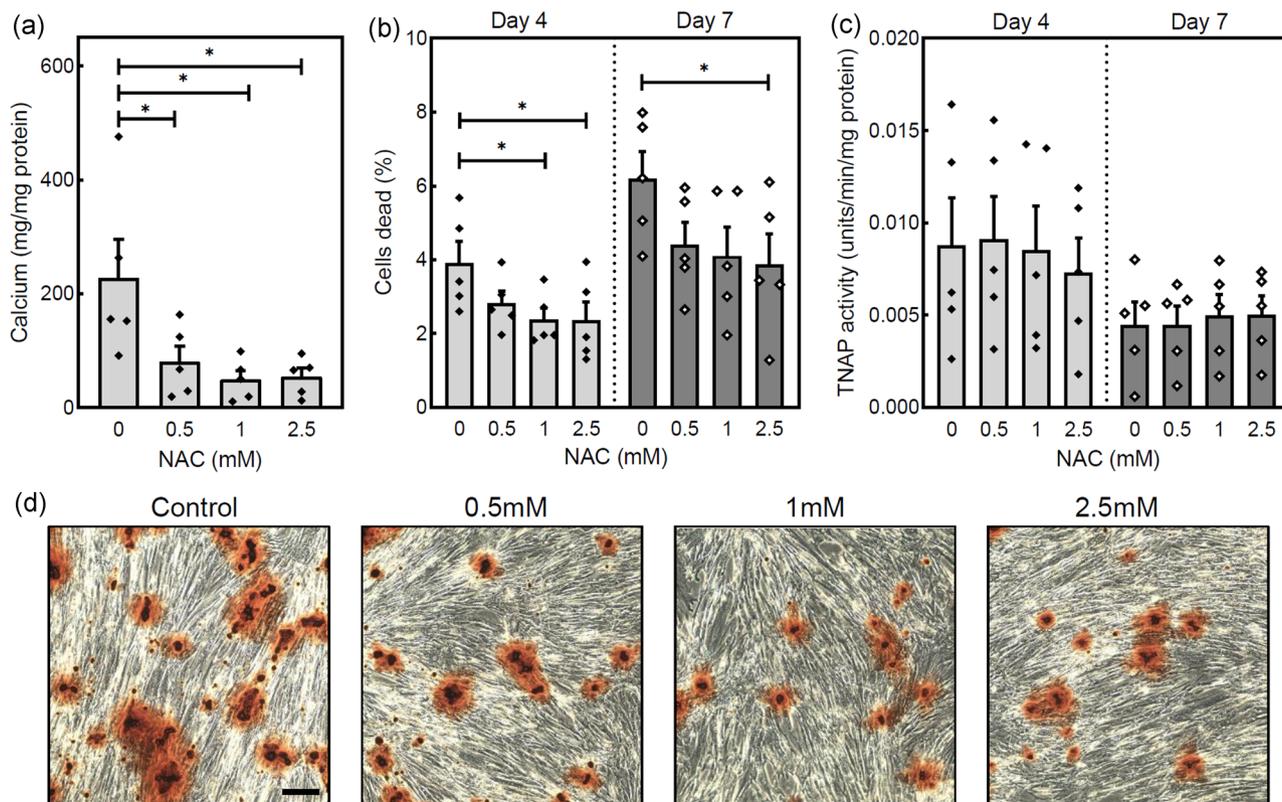


FIGURE 1 The effect of NAC on VSMC calcification, cell viability, and TNAP activity. (a) NAC (≥ 0.5 mM) dose-dependently decreases VSMC calcification by up to 80%. (b) NAC decreased VSMC cell death by up to 40% (c) but had no effect on TNAP activity. (d) Phase-contrast microscopy images show the reduced calcification in VSMC cultures treated with NAC. Scale bar = 200 μ m. Data shown as mean \pm SEM with points showing values for individual experiments ($n = 5$): * $p < 0.05$ compared with control. NAC, N-acetylcysteine; SEM, standard error of mean; VSMC, vascular smooth muscle cell

3.3 | The effect of NAC on mRNA and protein expression in calcifying VSMCs

To allow a targeted investigation of the downstream actions of NAC, this study examined the expression of key VSMC and osteoblast marker genes in calcifying VSMCs. NAC had no effect on the mRNA levels of VSMC genes, including *SM22 α* and *MYH11*; a trend towards increased expression of *Acta2* was

observed at Day 4 but this was not significant (Figure 3a–c). Whilst expression of several osteoblast-associated genes (*RUNX2*, *SPP1* [Opn]) was unchanged, NAC decreased *ALPL* (TNAP) expression (Figure 3d–f). Consistent with the qPCR data, NAC had no effect on the expression of Runx2, Opn, *SM22 α* , or α SMA protein expression (Figure 3g–i). The decreased mRNA expression for *ALPL* was not reflected at the protein level (Figures 3g and 3k).

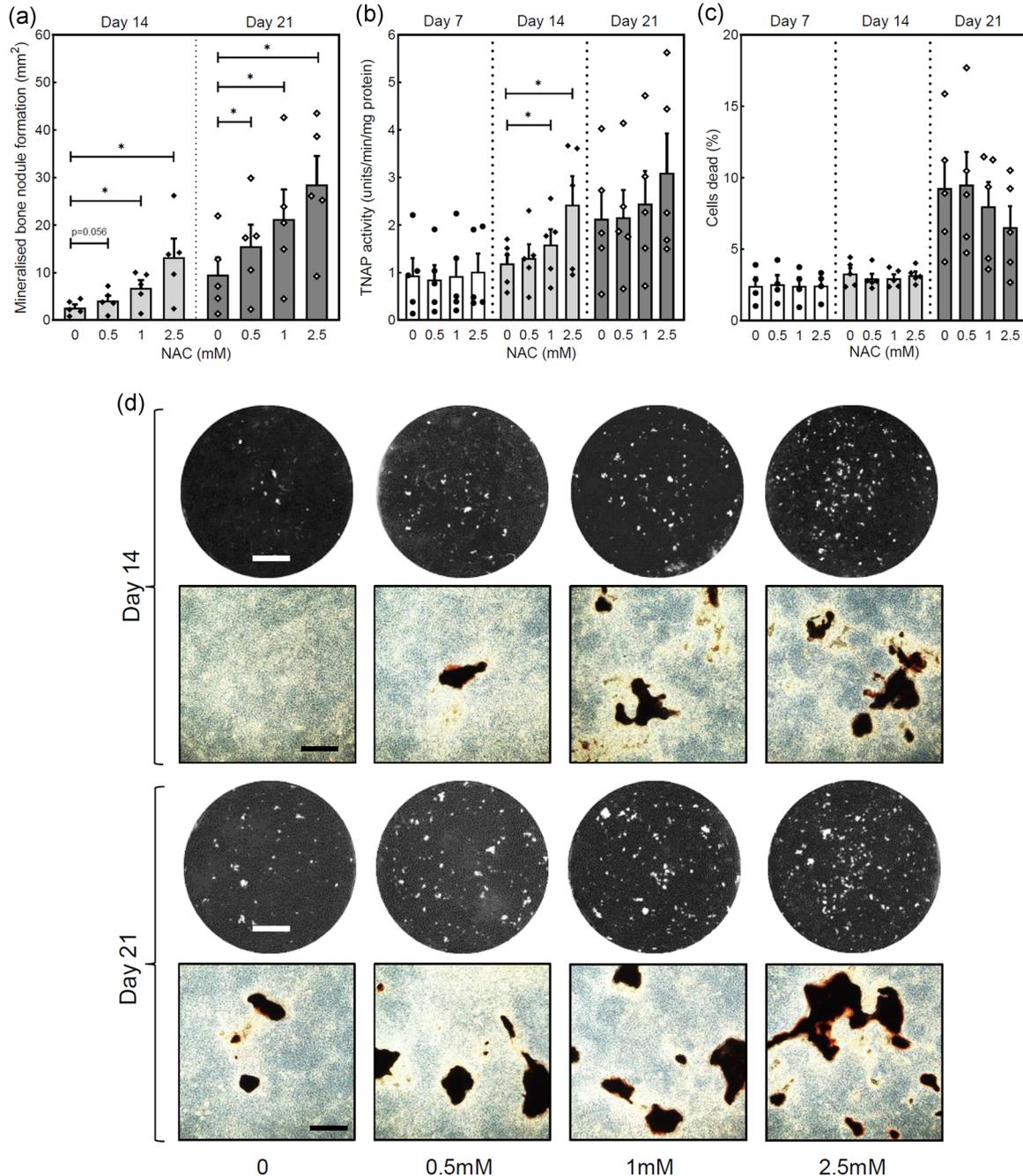


FIGURE 2 The effect of NAC on bone formation, TNAP activity, and osteoblast viability. (a) NAC (≥ 1 mM) increased the bone formation by up to 5- and 2-fold at Days 14 and 21, respectively. (b) TNAP activity was up to 2-fold higher in osteoblasts cultured with NAC. (c) NAC had no effect on viability at any stage. (d) Whole-well scans and light microscopy images show the increased bone formation seen in NAC-treated cells. Scale bars: whole well = 0.5 cm, microscopy = 200 μ m. Data shown as mean \pm SEM with points showing values for individual experiments ($n = 4-5$); * $p < 0.05$ compared with control. NAC, N-acetylcysteine; SEM, standard error of mean

3.4 | The effect of NAC on the expression of osteoblast markers

In osteoblasts, NAC (≥ 1 mM) increased expression of *Sp7* (≤ 4 -fold), *Bglap* (OCN) (≤ 6 -fold), and *Alpl* (≤ 4 -fold). *Runx2* mRNA expression was unchanged by NAC (Figure 4a–d). Conversely, NAC decreased the mRNA expression of genes associated with inhibiting mineralisation, namely *Spp1* and *Enpp1* (Figure 4e,f). NAC had no effect on *Runx2* and *Opn* protein expression (Figure 4g–i). NAC increased TNAP protein levels in proliferating cells (30%) but decreased expression (35%) in mature, mineralising cells (Figures 4g and 4j).

3.5 | NAC decreased AMC but had no effect on trabecular and cortical bone parameters in vivo

μ CT analysis of the whole vessel revealed that the development of AMC was not uniform; calcification was primarily observed in the aortic arch, the top of the descending aorta and the abdominal aorta down to the bifurcation; the thoracic aorta was typically devoid of calcification (Figure 5a). This pattern of AMC was consistent across all samples. Quantification showed that NAC had no effect in the upper aorta (aortic arch to the thoracic aorta) but decreased AMC by 25% ($p < 0.05$) in the abdominal aorta (Figure 5b,c). Representative images of AMC are shown in Figure 5a.

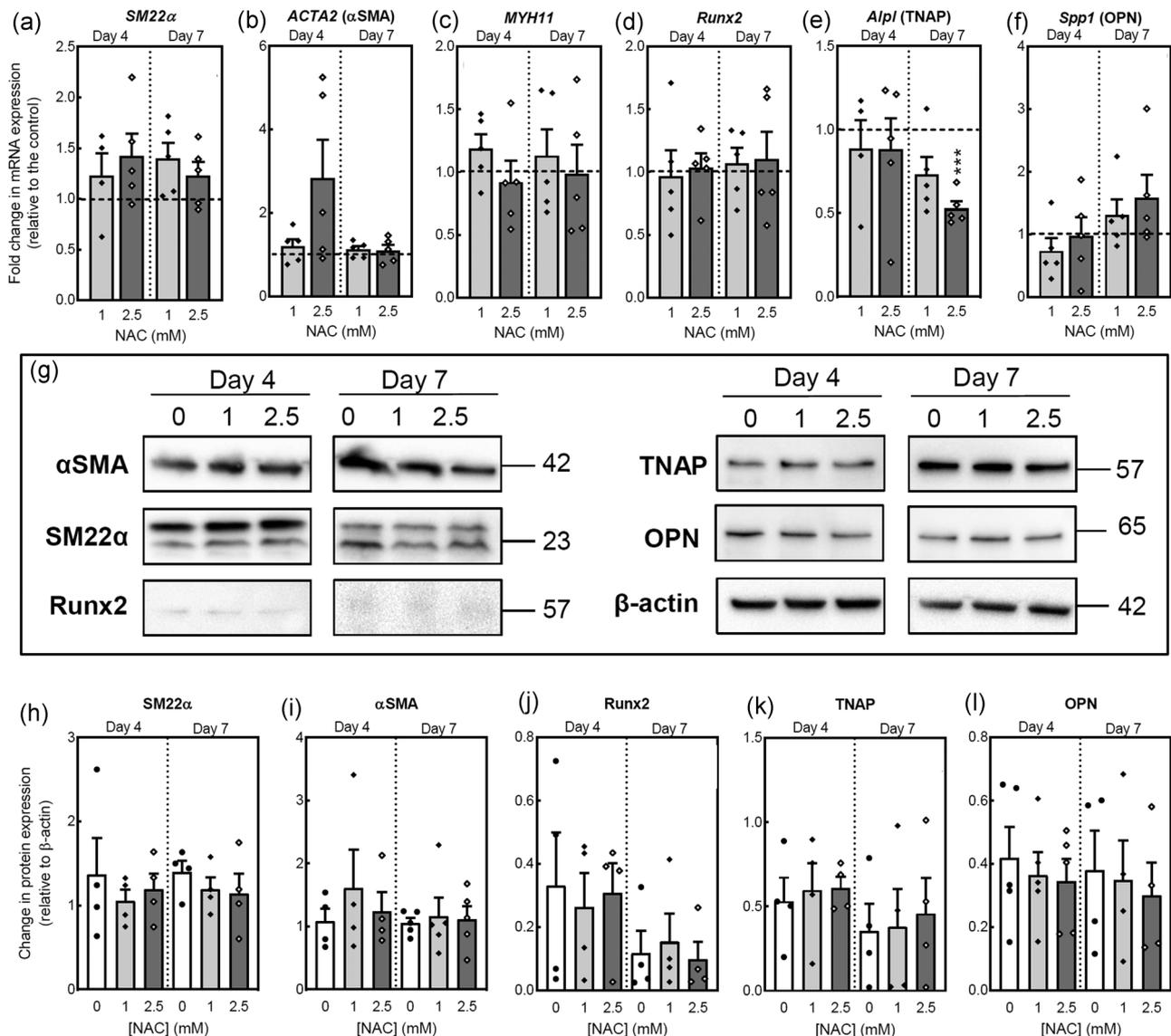


FIGURE 3 The effect of NAC on VSMC mRNA and protein expression. NAC did not influence the expression of typical (a–c) VSMC and (d–f) osteoblast marker genes; the only exception being a reduction in *ALPL* levels at Day 7. All gene expression was normalised to β -actin, calcifying control cells are represented by the dashed line. (g) NAC also had no effect on the protein expression of α SMA, SM22 α , Runx2, TNAP, or OPN. Blots shown are representative of the results obtained from 4 to 5 different protein sets. (h–l) Quantification of western blots showed no effect of NAC on protein expression (normalised to β -actin). Data shown as mean \pm SEM with points showing values for different RNA or protein sets ($n = 4$ –5), *** $p < 0.001$ compared with control. mRNA, messenger RNA; NAC, *N*-acetylcysteine; SEM, standard error of mean; VSMC, vascular smooth muscle cell

NAC treatment had no effect on the trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), bone mineral density (BMD), or cortical bone volume in the tibia or femur (Figure 5d–m). Representative images of trabecular and cortical bone from control and treated animals are shown in Figure 5n.

3.6 | The effects of L-cysteine on VSMC calcification and bone formation mimic those of NAC

It is well established in the literature that NAC is readily broken down to produce L-cysteine, an amino acid precursor for both GSH and H₂S (Figure 6a) (Dodd et al., 2008; Lasram et al., 2015). In osteoblasts,

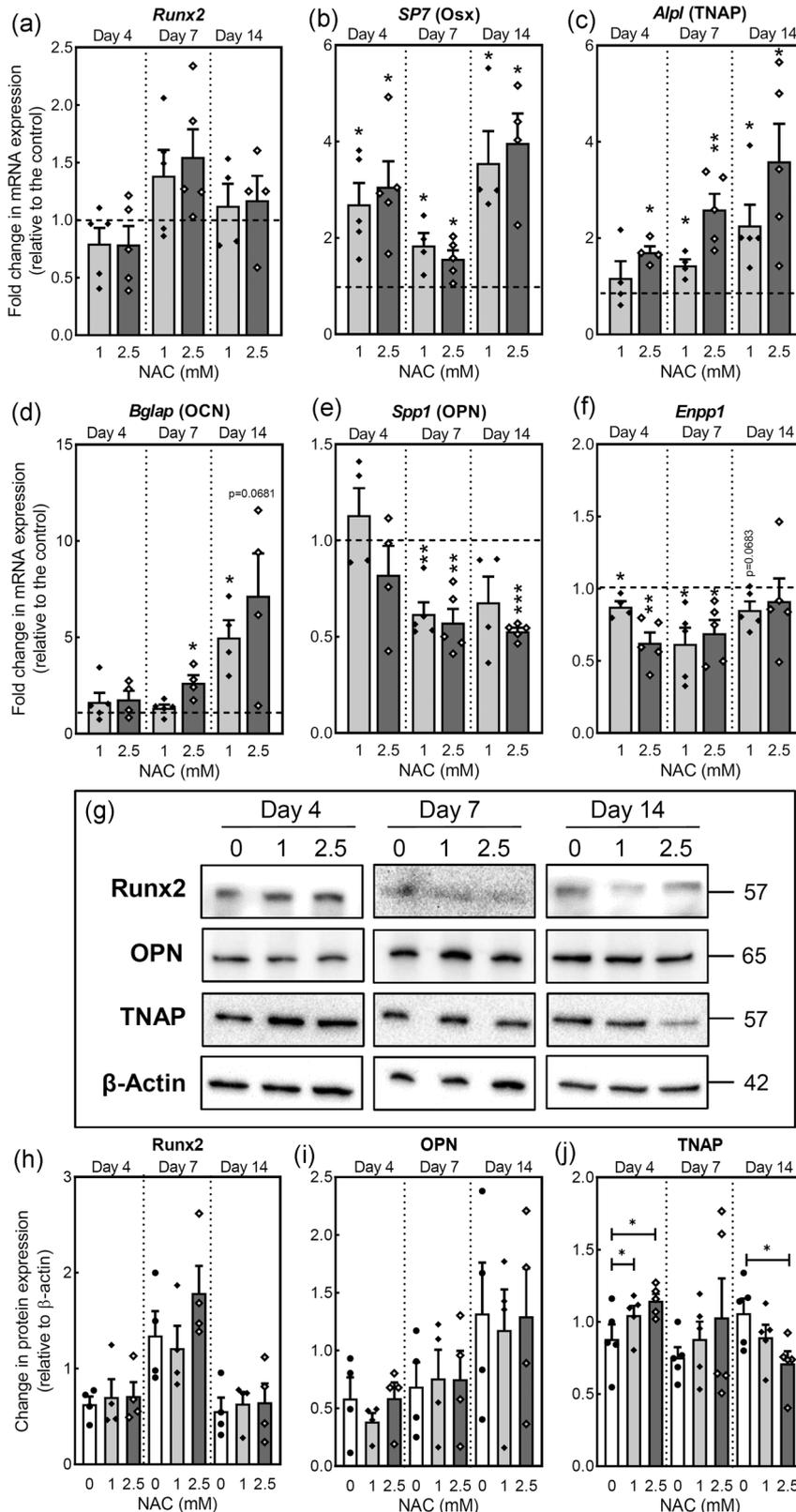


FIGURE 4 The effect of NAC on osteoblast mRNA and protein expression. (a–d) NAC increased the mRNA expression of *Sp7*, *Bglap*, and *Alpl* up to six-fold but had no effect on *Runx2* levels. (e) and (f) Expression of *Spp1* and *Enpp1* was decreased in NAC-treated osteoblasts. All gene expressions normalised to β -actin, control cells (untreated osteoblasts) are represented by the dashed line.

(g) Representative blots showing the effect of NAC on Runx2, OPN, and TNAP protein expression. Blots shown are representative of the results obtained from 4 to 5 different protein sets. (h–j) Quantification of western blots showed no effect of NAC on Runx2 and OPN expression; TNAP expression was increased in proliferating cells but decreased in mature mineralising cells (normalised to β -actin). Data shown as mean \pm SEM with points showing values for different RNA or protein sets ($n = 4$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control. mRNA, messenger RNA; NAC, N-acetylcysteine; SEM, standard error of mean

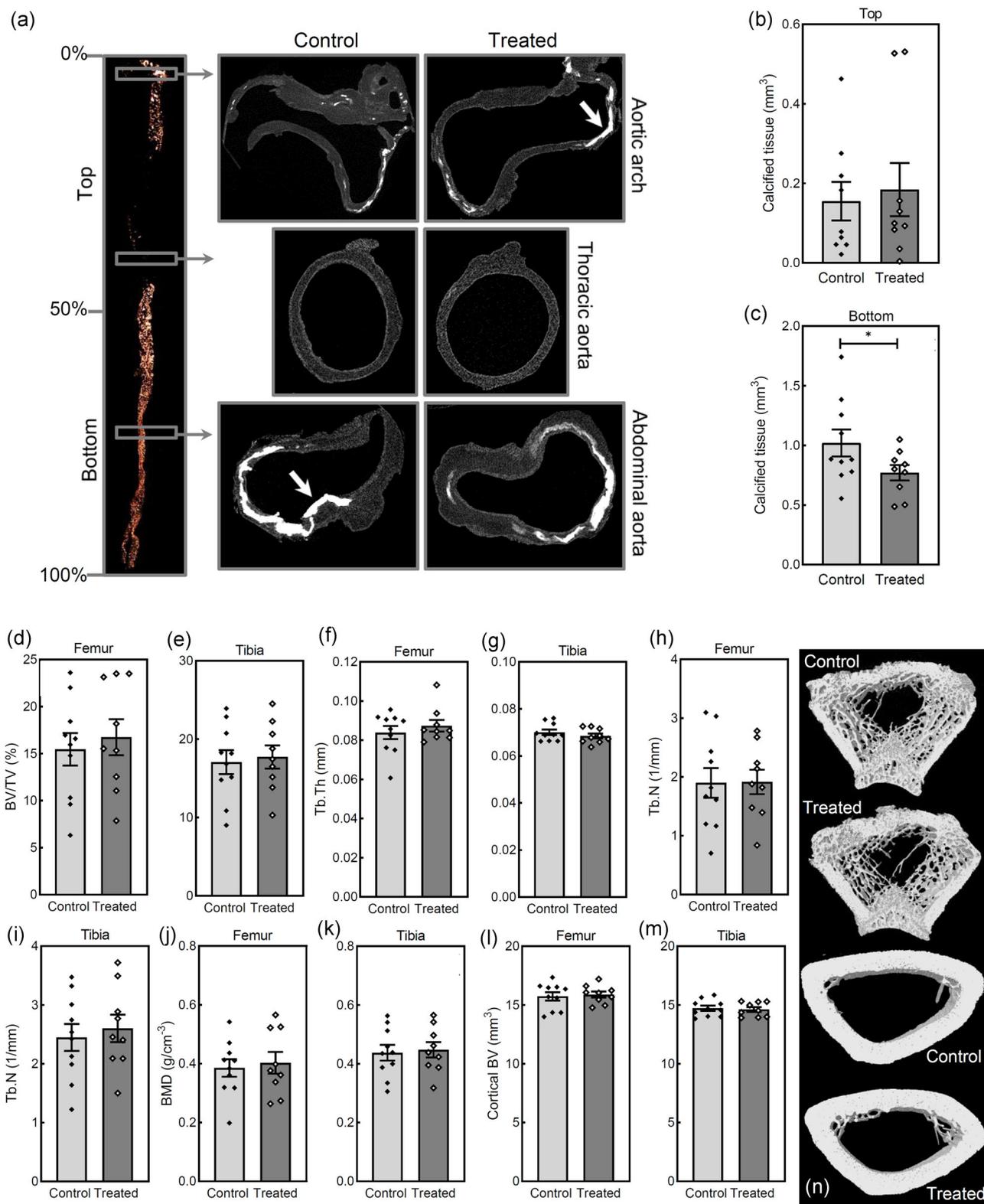


FIGURE 5 The effects of NAC on AMC and bone in vivo. (a) μ CT images showing the distribution of AMC in the rat aorta. (b) NAC had no effect on the amount of AMC in the upper aorta but (c) decreased AMC by 25% in the abdominal aorta. NAC had no effect on the (d) and (e) BV/TV, (f) and (g) Tb.Th, (h) and (i) Tb.N, (j) and (k) BMD or (l) and (m) cortical bone volume in the tibia or femur. (n) Representative images of the trabecular and cortical bone in control and NAC-treated animals. Data shown as mean \pm SEM with points for individual animals ($n = 9-10$): * $p < 0.05$ compared with untreated animals. AMC, arterial medial calcification; BMD, bone mineral density; BV/TV, trabecular bone volume; μ CT; microcomputed tomography; NAC, N-acetylcysteine; SEM, standard error of mean; Tb.N, trabecular number; Tb.Th, trabecular thickness

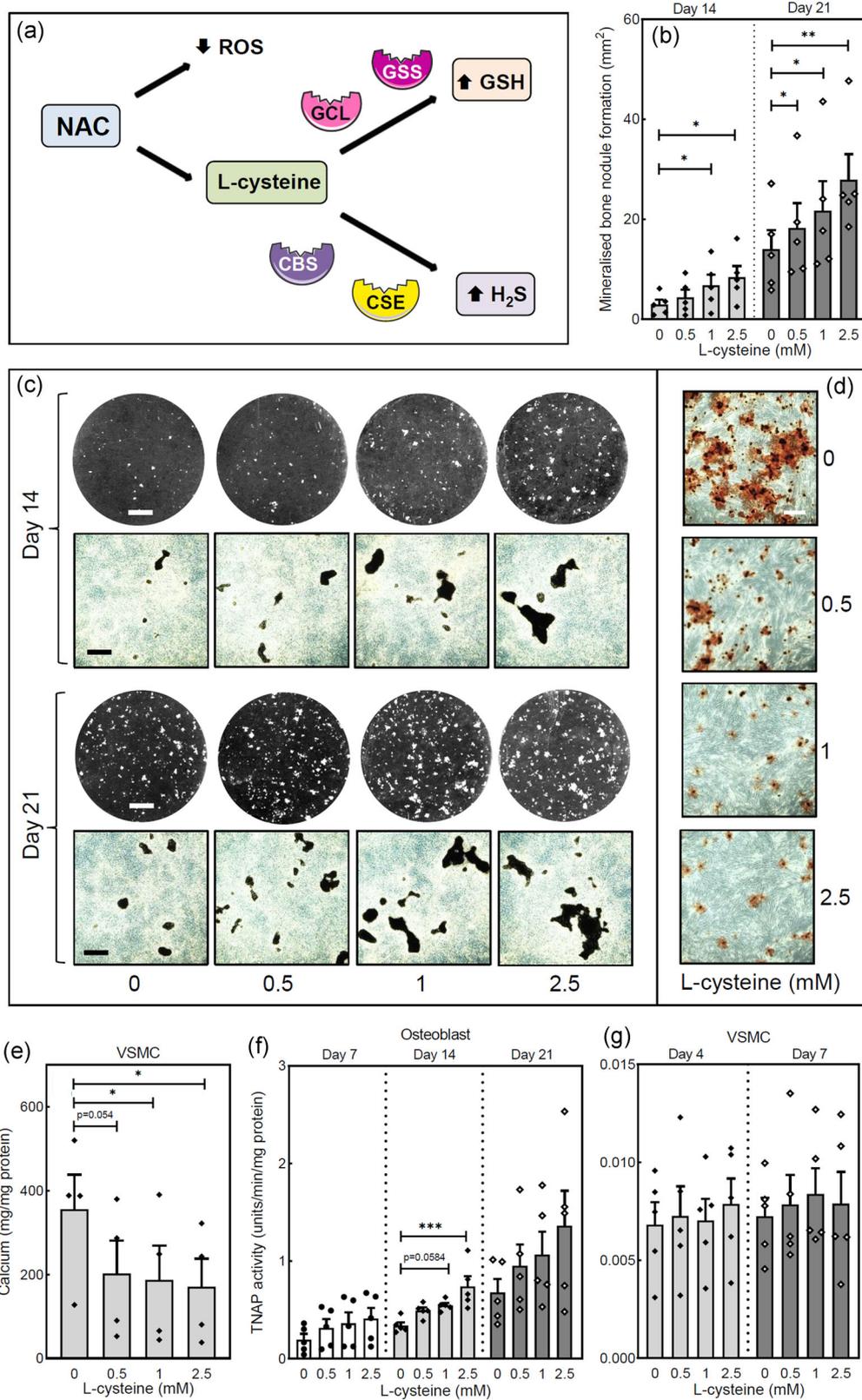


FIGURE 6 The effect of L-cysteine on VSMC calcification and bone formation. (a) Diagram showing the pathways via which NAC is metabolised to L-cysteine, GSH, and H₂S. (b) L-cysteine increased bone formation by up to 2.4-fold. Representative images of L-cysteine-treated (c) osteoblasts and (d) VSMCs. Scale bars: whole well = 0.5 cm, microscopy = 200 μm. (e) L-cysteine decreased VSMC calcification by up to 50%. (f) Osteoblast TNAP activity was increased up to two-fold by L-cysteine. (g) No effect of L-cysteine on VSMC TNAP activity. Data shown as mean ± SEM with points for individual experiments (n = 4–5); *p < 0.05, ***p < 0.001 compared with control. GSH, glutathione; NAC, N-acetylcysteine; SEM, standard error of mean; VSMC, vascular smooth muscle cell

L-cysteine (≥ 1 mM) increased bone formation by up to 2.8-fold ($p = < 0.05$) and up to two-fold ($p = < 0.01$) at Day 14 and Day 21, respectively (Figure 6b). Whole-well scans and images show that L-cysteine-treated cells produced bone nodules earlier and more extensively than control cells (Figure 6c). In VSMCs, L-cysteine reduced calcification by 48% and 50%, at 1 mM and 2.5 mM, respectively ($p = < 0.05$, Figure 6e). Representative alizarin red-stained images showing decreased calcification in L-cysteine-treated VSMCs are shown in Figure 6d. In mouse VSMCs, L-cysteine reduced calcification by 25% ($p = < 0.05$, Figure S1b).

L-cysteine (≥ 1 mM) increased TNAP activity up to 2.1-fold (Figure 6f) in mature osteoblasts; a trend towards increased activity was also observed in differentiating and mineralising cells. L-cysteine had no effect on TNAP activity in calcifying VSMCs (Figure 6g).

Analysis of gene expression in osteoblasts showed that L-cysteine increased the expression of *Sp7* (≤ 2.3 -fold), *Bglap* (≤ 2 -fold), and *Alpl* (≤ 2 -fold) whilst *Enpp1* mRNA levels were decreased (Figure S2a). In calcifying VSMCs, L-cysteine had no effect on gene expression, the only exception being an increase in *SPP1* mRNA levels (≤ 2.5 -fold) (Figure S2b).

3.7 | H₂S inhibits VSMC calcification while promoting bone formation

H₂S increased mineralised bone nodule formation by 50% and 60% at 150 μ M ($p = < 0.01$) and 300 μ M ($p = < 0.05$), respectively (Figure 7a). Conversely, H₂S (≥ 150 μ M) inhibited VSMC calcification by up to 40% ($p = < 0.05$, Figure 7b). Images showing the effects of H₂S on VSMC calcification and bone formation are shown in Figure 7c. In mouse VSMCs, H₂S reduced calcification by 25% (Figure S1c). Culture with H₂S (≤ 300 μ M) had no effect on gene expression in osteoblasts (Figure S2c). In calcifying VSMCs, H₂S downregulated *RUNX2* and *ALPL* expression in the early stages of culture (Figure S2d).

3.8 | The effects of NAC and L-cysteine are unlikely to be mediated by increased H₂S levels

Calcifying VSMCs and osteoblasts both express the two main enzymes involved in H₂S generation, CBS and CSE (Figure S3). NAC and L-cysteine had no effect on the mRNA and protein expression of these enzymes in either cell type (Figure S3). Broad-spectrum pharmacological inhibitors of CBS (AOAA) and CSE (PAG) were co-administered with NAC and L-cysteine. Neither inhibitor blocked the ability of NAC or L-cysteine to promote bone formation (Figure 7d–g) or inhibit VSMC calcification (Figure 7h–k).

The fluorescent probe SF₇-AM was used to visualise endogenous H₂S production in calcifying VSMCs and osteoblasts. Figure 7l shows that both cell types can produce H₂S under basal conditions and the addition of NaHS as a positive control lead to increased fluorescence. Qualitative observations showed that the addition of NAC or

L-cysteine had no effect on the fluorescence level and hence the level of H₂S production (Figure 7l).

3.9 | NAC and L-cysteine increase GSH levels

NAC (2.5 mM) increased GSH levels in proliferating osteoblasts by 3.4-fold ($p = < 0.01$) and in differentiating cells by 60% ($p = < 0.05$). At the same time points, L-cysteine enhanced GSH by 3.2-fold ($p = < 0.01$) and 53% ($p = < 0.05$). Neither compound influenced GSH levels in mature, bone forming cells (Figure 8a). In calcifying VSMCs (Day 7), NAC and L-cysteine increased GSH levels by up to 2.2-fold ($p = < 0.001$) and 1.9-fold ($p = < 0.01$), respectively (Figure 8b).

3.10 | GSH inhibits VSMC calcification and promotes bone formation

In osteoblasts, GSH (1–10 μ M) increased mineralised bone nodule formation by up to 1.9-fold ($p < 0.001$). No effects were seen at 100 μ M but this level of GSH was associated with a reduction in medium pH (Figure 8c). Culture with 100 μ M GSH decreased VSMC calcification by 65% (Figure 8d).

4 | DISCUSSION

This study demonstrated that NAC has beneficial but divergent effects on VSMCs and osteoblasts. Specifically, NAC can promote osteoblast differentiation and bone formation but inhibit VSMC calcification. L-cysteine mimicked the actions of NAC suggesting that metabolites of NAC mediate the functional effects observed. Whilst H₂S also exerted the same effects on VSMC calcification and bone formation, these were less efficacious than NAC or L-cysteine. Mechanistic analysis suggests that the actions of NAC and L-cysteine do not occur via metabolism to H₂S but instead involve increased GSH synthesis. Combined, these comparative data show that whilst the intracellular processes mediating the effects of NAC and L-cysteine on osteoblasts and VSMCs appear similar, the overall downstream effects on mineralisation/calcification processes are opposite.

The data presented here show that NAC can inhibit VSMC calcification in a dose-dependent manner. The development of AMC is multifactorial and includes phenotypic changes within VSMCs and increased apoptosis (Bourne et al., 2021; Durham et al., 2018). This study found that, despite inhibiting calcification, NAC did not attenuate the changes in marker gene expression or TNAP activity caused by high phosphate; however, NAC did markedly reduce VSMC cell death. This suggests that the effects of NAC on calcification are likely independent of any phenotypic changes and instead a consequence of improved cell survival. In agreement, previous studies have shown that pharmacological inhibition of apoptosis can decrease VSMC calcification (Ciceri et al., 2016; Patel et al., 2018; Patel

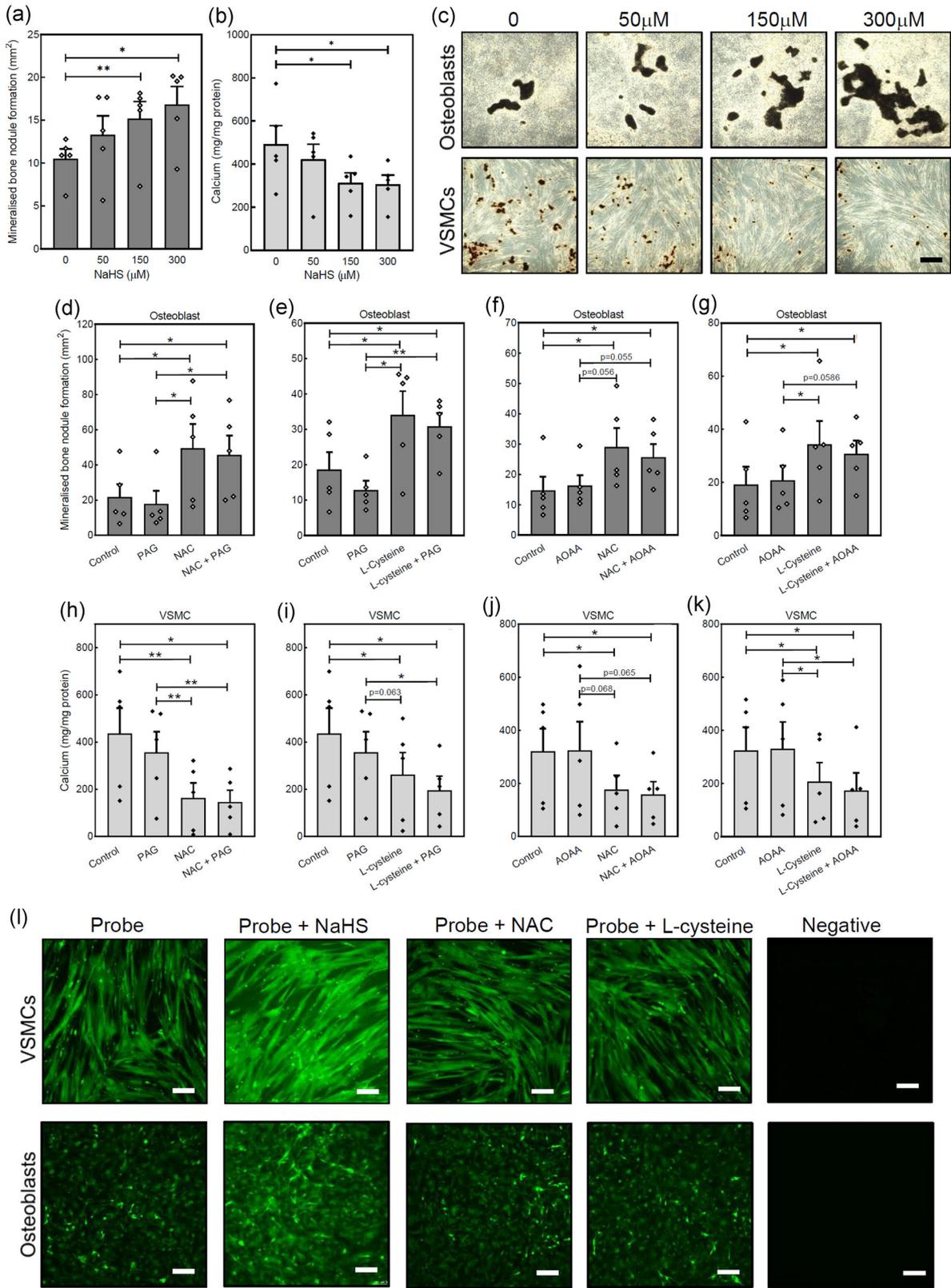


FIGURE 7 The effect of H₂S on VSMC calcification and bone formation. (a) Bone formation was increased up to 60% in osteoblasts cultured with H₂S (as NaHS) (b) H₂S decreased VSMC calcification by up to 40%. (c) Light microscopy images showing the effects of H₂S on bone formation and VSMC calcification. Scale bar = 200 μm. (d–g) The CSE inhibitor, PAG, and the CBS inhibitor, AOAA, did not prevent the stimulatory actions of NAC and L-cysteine on bone formation. (h–k) PAG and AOAA had no effect on the ability of NAC and L-cysteine to inhibit VSMC calcification. (l) Representative immunofluorescence images showing that the addition of NAC and L-cysteine did not increase H₂S production in VSMCs or osteoblasts. Scale bar = 100 μm. Data shown as points for individual experiments (n = 4–5): *p < 0.05, **p < 0.01, ***p < 0.001 compared with control. AOAA, aminooxyacetic acid; CBS, cystathionine-β-synthase; CSE, cystathionine-γ-lase; NAC, N-acetylcysteine; PAG, DL-propargylglycine; VSMC, vascular smooth muscle cell

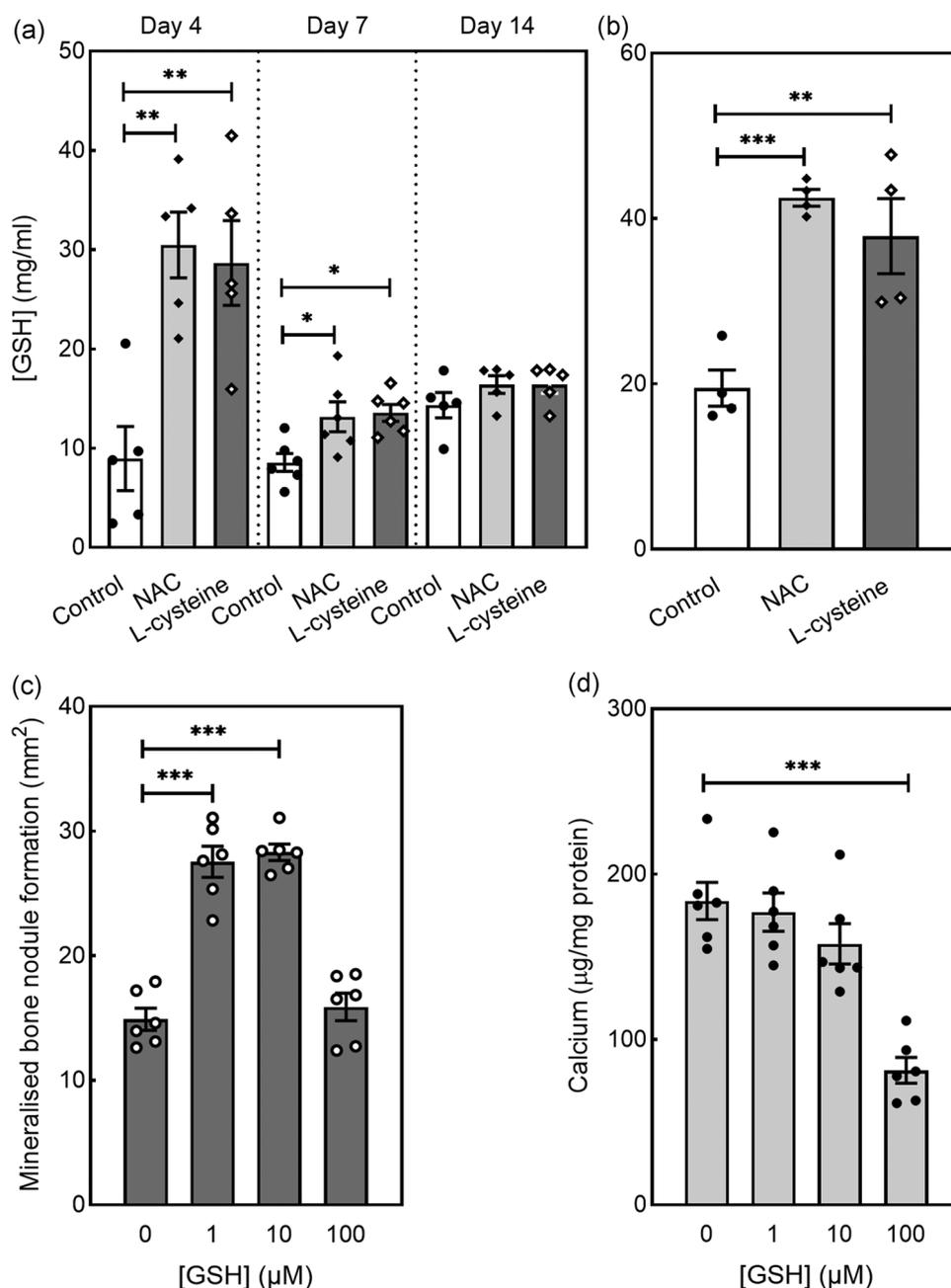


FIGURE 8 NAC and L-cysteine increase GSH levels in osteoblasts and VSMCs. (a) GSH levels were increased up to 3.4-fold in osteoblasts cultured with NAC and L-cysteine. (b) In calcifying VSMCs (Day 7), NAC and L-cysteine increase GSH levels by up to 2.2-fold. (c) Bone formation was increased 1.9-fold in osteoblasts treated with 1–10 μM GSH. (d) Culture with GSH decreased VSMC calcification by 65%. Data shown as mean ± SEM with points for individual experiments ($n = 4–6$): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control. GSH, glutathione; NAC, N-acetylcysteine; SEM, standard error of mean; VSMC, vascular smooth muscle cell

et al., 2019b). Furthermore, earlier work has reported that calcification can occur without changes in VSMC marker gene expression (Alves et al., 2014; Ter Braake et al., 2018).

In osteoblasts, NAC promoted osteoblast differentiation, leading to earlier and increased levels of bone formation. This is consistent with previous studies that reported beneficial actions of NAC on osteoblasts or osteoblast-like cells (Jun et al., 2008; Romagnoli et al., 2013; Yamad et al., 2013). While NAC increased expression of the

osteogenic genes osterix, TNAP and OCN, Runx2 expression was unchanged. The primary cells used here are already committed to the osteogenic lineage when isolated and so Runx2 is likely of lesser importance in terms of promoting osteoblast differentiation, thus potentially explaining the lack of effect of NAC on Runx2 expression. However, it remains possible that any changes in Runx2 expression could have happened before the first experimental time point at Day 4.

Interestingly, we found positive effects of NAC at much lower concentrations (≤ 2.5 mM) compared with the earlier work where levels of up to 30 mM were used. This could reflect the lower level of phosphate used to induce bone formation (2 mM vs. 10 mM) in this study. High levels of β -glycerophosphate lead to decreased osteoblast viability and widespread, dystrophic mineral deposition that is not true bone formation (Taylor et al., 2014). Thus, it is possible that in this situation a higher NAC concentration was required to visualise the beneficial effects. Jun et al. (2008) suggested that NAC was most important at the early stages of culture and adding it once bone formation had commenced had little effect. This indicates that NAC is primarily acting to promote osteoblast differentiation, resulting in an earlier onset of bone formation. This is consistent with our findings that NAC exerts greater effects on bone formation, TNAP activity and gene expression during the differentiation and onset of mineralisation phases of the culture. TNAP expression is temporal in osteoblasts and reaches a peak immediately before the onset of mineralisation (\sim Day 14) (Stein et al., 1990). Consistent with a faster differentiation, we observed higher TNAP activity in earlier stage cultures treated with NAC; the reduced expression at Day 14 most likely reflects the NAC-treated cells having passed the stage of peak TNAP expression.

Under normal conditions, rats do not develop AMC; however, when maintained on a warfarin-vitamin K1 diet they develop widespread AMC within approximately 8 weeks (Neven et al., 2015). This model of AMC was chosen to investigate the effect of NAC on vascular and bone parameters *in vivo* because it represents the most established and characterised approach to address our research questions. Here, daily dosing with NAC caused a 25% decrease in AMC but this was in a site-specific manner, only being evident in the abdominal aorta. No effects on trabecular or cortical bone were observed. This lack of effect on the bone *in vivo* is inconsistent with the *in vitro* osteoblast data. However, since NAC was administered orally at a relatively low dose it is possible that the concentration experienced by osteoblasts *in vivo* was not enough to induce osteogenic effects. In agreement, several studies using higher dosages have reported that NAC has beneficial effects on the skeleton although these have also used different experimental models (e.g., ovariectomy-induced bone loss) (Chen et al., 2019; Li et al., 2017; Zhou et al., 2020). While there are no studies describing the effects of NAC on AMC, previous work has suggested that NAC can have beneficial effects on calcification in the context of atherosclerosis (Ivanovski et al., 2005; Lin et al., 2015). These data collectively suggest that NAC may have beneficial effects *in vivo* to reduce AMC without exerting negative effects on the skeleton. The dosage of NAC administered in this study was lower than those used clinically (600 mg–6 g/day), therefore further studies in rodent models of AMC are required to optimise the dose and duration of NAC treatment to fully evaluate its beneficial effects *in vivo*.

This study provides the first description of the direct functional effects of L-cysteine on osteoblasts and calcifying VSMCs. Like NAC, L-cysteine promoted osteoblast differentiation, TNAP activity, and bone formation. It also inhibited VSMC calcification but had minimal effects on VSMC transdifferentiation and TNAP activity. Together these results suggest that NAC itself is unlikely to be mediating the beneficial actions observed but instead requires metabolism to L-cysteine. These findings are broadly in agreement with clinical

studies, which have reported an association between low plasma L-cysteine levels and reduced bone mineral density (Baine et al., 2007; Zhao et al., 2018) and *in vitro* work, which showed that the cysteine prodrug, 2-oxothiazolidine-4-carboxylic acid, inhibits VSMC calcification (Patel et al., 2021).

NAC, via breakdown to L-cysteine, could also act indirectly to increase the levels of H₂S. Here, and consistent with previous studies (Behera et al., 2018; Grassi et al., 2016; Liu et al., 2014; Zavadzki et al., 2011), H₂S was also found to promote bone formation but inhibit VSMC calcification. However, the effects of H₂S were less efficacious than those of NAC or L-cysteine. Both cell types also showed high expression of CBS and CSE, the levels of which were not modulated by NAC or L-cysteine. Pharmacological inhibition of these enzymes failed to attenuate the beneficial effects of either compound on bone formation or VSMC calcification. Additionally, treating osteoblasts or calcifying VSMCs with NAC or L-cysteine did not detectably increase endogenous H₂S levels. Together, these data suggest that whilst H₂S has beneficial effects on the bone and vasculature it is unlikely to be mediating the observed actions of NAC and L-cysteine. However, it is acknowledged that, as with most pharmacological inhibitors, the compounds used to inhibit CBS and CSE are not selective. Thus, to fully exclude the involvement of H₂S, loss of function approaches would also be required.

GSH is a fundamental antioxidant product of L-cysteine that is involved in scavenging ROS, detoxification of xenobiotics and protein thiolation (Rushworth & Megson, 2014). Previous work demonstrated that calcifying VSMCs display reduced GSS and GCL expression and GSH levels (Patel et al., 2021). Furthermore, increased GSH levels have been implicated in the beneficial actions of NAC on bone formation (Jun et al., 2008). This study found that in both osteoblasts and VSMCs GSH levels were increased by NAC and L-cysteine. Furthermore, reduced GSH, which is the form utilised by cells, had the same divergent effects, albeit at different concentrations, on bone formation and VSMC calcification as NAC and L-cysteine. It has been suggested previously that the actions of NAC on VSMC calcification and bone formation are mediated via direct inhibition of ROS (Balogh et al., 2019; Jun et al., 2008; Sun et al., 2019). The effect of NAC on ROS was not measured here, and so reduced levels could contribute to the beneficial effects observed. However, since L-cysteine mimics the actions of NAC, direct ROS scavenging is unlikely to be the primary mechanism of action. Instead, our findings suggest it is the higher levels of GSH (and not H₂S) that most likely mediates the effects of NAC and L-cysteine on bone formation and VSMC calcification.

It is widely accepted that in a pro-calcifying environment VSMCs undergo several phenotypic changes to make them more osteoblast-like, yet few studies look at these cells in parallel. By undertaking a detailed comparative analysis, this investigation adds to the growing body of evidence that shows there are fundamental differences between calcifying VSMCs and osteoblasts (Alves et al., 2014; Mody et al., 2001; Patel et al., 2018; Patel et al., 2019a). Here, VSMCs and osteoblasts were cultured using identical tissue culture conditions; the novel combination of 1 mM β -glycerophosphate and 1 mM sodium diphosphate results in cell-mediated calcification and does not cause dystrophic mineral deposition, a common occurrence when

excessive levels of phosphate are used (Patel et al., 2019a; Taylor et al., 2014). Thus, the divergent effects observed in this study are unlikely to be a consequence of culture conditions. It is acknowledged that the VSMCs and osteoblasts used were primarily from different species being human and mouse, respectively. The actions of NAC, L-cysteine and H₂S were also measured in mouse aortic VSMCs and all three compounds were found to be inhibitory. Isolation of mouse VSMCs requires a very large number of animals, something which is inconsistent with the drive to reduce animal usage in research. Therefore, given that the same functional effect was observed in human VSMCs, it was decided to use these cells for the mechanistic analysis. Primary human osteoblasts are difficult to obtain and are usually from aged or diseased patients (Taylor et al., 2014); therefore, to avoid potentially confounding results we opted to use mouse osteoblasts. Our previous work has shown that osteoblasts from different species respond to endogenous or exogenous compounds in a similar manner (Orriss et al., 2007; Patel et al., 2018). Together, these data show that species variations are unlikely to cause the divergent effects observed.

In conclusion, this study showed that NAC, via breakdown to L-cysteine, has opposing actions on pathological VSMC calcification and physiological bone formation. Furthermore, whilst the molecular mechanisms driving the actions of NAC in osteoblasts and VSMCs appear to be comparable, the downstream cellular effects are very different. In osteoblasts, NAC increases differentiation and bone formation but has no effect on cell survival. On the other hand, NAC decreases cell death and calcification in VSMCs but does not influence gene expression. Our findings also agree with a number of studies, which have shown that ROS and antioxidants exert differential effects on VSMC calcification or bone formation (Cui et al., 2017; Dai et al., 2007; Hou et al., 2016; Orriss et al., 2016; Pang et al., 2018; Zhang et al., 2016). Thus, NAC could represent a therapeutic option for treating AMC without exerting deleterious effects on the skeleton.

ACKNOWLEDGEMENTS

The authors acknowledge the British Heart Foundation (grant number: PG/15/13/31296) and the Rosetrees Trust (grant number: M744) for funding this study. We are also grateful to the RVC statistician, Dr Yu-Mei Chang, for assistance in the statistical analysis of all the data presented.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Lucie E. Bourne performed all the in vitro experimental work presented in Figures 1–4, 6–8, and S1–S3, with initial training on cell isolation from Jessal J. Patel and subsequent assistance from Bethan K. Davies and Isabel R. Orriss. The in vivo study in Figure 5 was performed by Jessal J. Patel with support from Lucie E. Bourne and Isabel R. Orriss. Ellen Neven, Anja Verhulst, and Patrick C. D'Haese provided technical advice, support, and training for the work

presented in Figure 5. The project was conceptualised and designed by Lucie E. Bourne, Jessal J. Patel, Caroline P. D. Wheeler-Jones, and Isabel R. Orriss. Funding to perform the work was obtained by Caroline P. D. Wheeler-Jones and Isabel R. Orriss. The manuscript was written by Lucie E. Bourne and Isabel R. Orriss. All authors reviewed and commented on the completed manuscript.

ORCID

Isabel R. Orriss  <https://orcid.org/0000-0002-7847-050X>

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How to cite this article: Bourne, L. E., Patel, J. J., Davies, B. K., Neven, E., Verhulst, A., D'Haese, P. C., Wheeler-Jones, C. P. D., & Orriss, I. R. (2021). N-acetylcysteine (NAC) differentially affects arterial medial calcification and bone formation: The role of L-cysteine and hydrogen sulphide. *Journal of Cellular Physiology*, 1–17. <https://doi.org/10.1002/jcp.30605>