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ORIGINAL ARTICLE

Inhibitory effects of orthosilicic acid on osteoclastogenesis in RANKL-stimulated RAW264.7 cells

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Abstract

Numerous studies have reported on the positive effects of silicon (Si) on bone metabolism, particularly on the stimulatory effects of Si on osteoblast cells and on bone formation. Inhibitory effects of Si on osteoclast formation and bone resorption have also been demonstrated in vitro and are suggested to be mediated indirectly via stromal and osteoblast cells. Direct effects of Si on osteoclasts have been less studied and mostly using soluble Si, but no characterisation of the Si treatment solutions are provided. The aims of the present study were to (a) further investigate the direct inhibitory effects of Si on osteoclastogenesis in RANKL-stimulated RAW264.7 cells, (b) determine at what stage during osteoclastogenesis Si acts upon, and (c) determine if these effects can be attributed to the biologically relevant soluble orthosilicic acid specie. Our results demonstrate that silicon, at 50 μg/ml (or 1.8 mM), does not affect cell viability but directly inhibits the formation of TRAP+ multinucleated cells and the expression of osteoclast phenotypic genes in RAW264.7 cells. The inhibitory effect of Si was clearly associated with the early stages (first 24 hr) of osteoclastogenesis. Moreover, these effects can be attributed to the soluble orthosilicic acid specie.

KEYWORDS
bioceramics, bone metabolism, orthosilicic acid, osteoclast differentiation, RAW264.7 cells

1 | INTRODUCTION

Impaired bone metabolism and remodelling, due to age or disease, may affect healing and reconstruction of bone after pathologies or traumatic injuries. Positive effects on bone by dietary components, such as calcium and vitamin D, are well documented. Along the same lines, there is increasing interest in other nutritional factors for optimal skeletal health.[1] In 1970s, animal experiments demonstrated the potential importance of silicon (Si) in skeletal and connective tissue development and growth.[2,3] Although the highly dramatic effects reported in these studies have not been since replicated, there are accumulating evidence for positive beneficial effects of Si on bone metabolism.[4-9] Silicon is ubiquitous in nature and is found combined with oxygen in the form of silica/silicates.[10,11] Weathering of rocks release soluble orthosilicic acid (OSA, $Si[OH]_4$, monomeric silica) into natural waters and drinking waters.[11] Indeed, orthosilicic acid is the predominant Si specie in aqueous solutions with Si concentrations <56 μg/ml. At concentrations >56 μg/ml, $Si(OH)_4$ starts to polymerise (auto condense) to form polymers that have reduced solubility and bioavailability.[11]

Dietary Si is the main source of Si exposure for the general population. Orthosilicic acid is the only known Si specie that is readily

absorbed by humans from the diet.[10] Plant based diets and beverages accounts for the main dietary sources of exposure to Si.[12] In food, Si generally exists as polymers of different complexities that require hydrolysis to OSA in the gastrointestinal tract prior to intestinal uptake.[13] The amount of bioavailable Si in food therefore varies depending on the complexity of the polymers present and other dietary components.[13] Upon gastrointestinal uptake, OSA is readily absorbed into blood and excreted in urine.[13] In healthy non-pregnant humans, fasting serum Si concentration is approximately 0.2 mg/L and 24-hr urinary Si excretion is approximately 8 mg Si/day.[14-16] The physiological role of Si (OSA) in humans and other mammals is yet to be established. The recent identification of transport mechanisms for Si (OSA) in mammals suggests some form of control of Si homeostasis and thereby implies an important role for Si in mammals.[17,18]

Silicon is also a common component of bone graft materials such as Bio-Oss (from bovine bone) and Bioglass (synthetic) that are used in the reconstruction of craniofacial and dentoalveolar bone defects.[19,20] The amount of Si in Bio-Oss is batch-dependent, which may reflect the variability in the animal's exposure to dietary Si.[19] Bioglass 45S5 with the composition of 45% SiO_2, 25% Na_20, 27% CaO, and 3% P_2O_5 consists of small glass particles that are gradually resorbed and replaced by bone at the reconstruction site.[20,21] In cell culture medium, Bio-Oss and bioactive glasses release significant amounts of Si (up to 350 μg/ml), which suggests high levels of Si are released at the site of implantation in bone reconstruction.[19,20,22] However, it is not known what form of Si (soluble OSA or particulate Si) is released from the biomaterials into the cell culture medium. As Bio-Oss and bioactive glass also change the levels of calcium and phosphate in the cell culture medium, in vitro cell culture studies have been carried out to investigate the effects of Si alone on bone cells.[22-24] The majority of studies investigating the impact of Si on bone metabolism have focused on the widely demonstrated favourable osteogenic response. Silicon, in the same range of concentrations (15–20 μg/ml) that are released from Bioglass 45S5, increases the expression of bone formation markers, extracellular matrix synthesis, and the formation of mineralised nodules in human foetal osteoblasts.[25] Physiological concentrations of Si (0.3–0.6 μg/ml) also increases osteogenic differentiation in cultured mouse and human osteoblast-like cells, and in human mesenchymal stem cells.[23,24,26] There are only a few studies demonstrating the effect of Si on osteoclasts and bone resorption. Silica/silicate increases the expression of osteoprotegerin in SaOS-2 osteosarcoma cells and inhibits osteoclastogenesis in a co-culture system with receptor activator of nuclear factor κ-B ligand (RANKL) stimulated RAW264.7 cells.[27] Silicon also inhibits the expression of macrophage colony stimulating factor (MCS-F) and RANKL in cultured human mesenchymal stem cells.[24] These findings support the hypothesis that stromal/osteoblast cells may mediate the inhibitory effects of Si on osteoclastogenesis and on bone resorption. However, there are also evidence for a direct effect of Si on osteoclasts. We have previously, in 2014, reported that Si inhibits bone resorption in vitro and dose-dependently reduced the number of TRAP+ multinucleated cells that were differentiated from bone marrow cultures and cultures of RAW264.7 cells.[22] This inhibitory effect of Si on osteoclastogenesis has been confirmed by others, in stimulated hCD14+ osteoclast precursors and in mouse bone marrow macrophages.[24,28] However, mechanisms have not been established. It is also not known at what stage(s) of osteoclastogenesis Si acts on to inhibit osteoclast differentiation. Moreover, it is not clear which form of Si (soluble, polymeric/particulate, or both) is responsible for the inhibitory effect. Beck et al., reported their findings of stimulative effects on osteoblast differentiation and suppressive effects on osteoclast differentiation using engineered nano-particulate silica (50 nm).[29] However, most studies report using soluble Si (silicic acid or OSA), but provide only brief details of their preparation and no characterisation of the Si treatment solution.[22,24,28] Those with knowledge of aqueous Si chemistry will acknowledge its complexity and that transient polymeric/particulate Si species can readily and easily be formed, especially in tissue culture media, when the soluble, and biologically relevant, OSA specie is intended.

The aims of the present study were to (a) further investigate the direct inhibitory effects of Si on osteoclastogenesis, (b) specifically determine at what stage in the osteoclastogenesis Si acts on, and (c) determine whether it is soluble or particulate Si that is responsible for the inhibitory effects. In the present study, we used RAW264.7 cells, an established cell line that does not require co-culture with mesenchymal/osteoblast cells for osteoclast formation.[30,31] RANKL was used to stimulate osteoclastogenesis in RAW264.7 cells and this was confirmed by the expression of osteoclast markers (*RANK*, *TRAP*, *CtsK*, and *CalcR*).

2 | MATERIAL AND METHODS

2.1 | Si-supplemented cell culture medium

2.1.1 | Preparation of Si-supplemented cell culture medium

Standard preparation of the Si-supplemented cell culture medium was according to previously published protocol.[22,32] Briefly, a stock 350 μg/ml Si solution was prepared by adding 100 μl of concentrated basic sodium silicate solution (reagent grade, Sigma-Aldrich, St. Louis, MO) to 49.9 ml minimum essential medium α (α-MEM; Gibco, ThermoFisher, Waltham, MA) supplemented with 1% antibiotic–antimycotic (Anti-Anti; Gibco, ThermoFisher, Waltham, MA) and 1% L-alanyl-L-glutamine (GlutaMAX; Gibco, ThermoFisher, Waltham, MA). The pH was immediately adjusted to 7.0–7.2 with concentrated HCl (ACS reagent 37%, Sigma-Aldrich, St. Louis, MO) and then supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco, ThermoFisher, Waltham, MA). This stock Si solution was then immediately diluted (within 10 min of preparation) in cell culture media (α-MEM supplemented with antibiotic/antimycotic, glutamine and 10% FBS) to the desired Si concentrations (25–150 μg/ml Si) and left to stand for 24 hr before addition to the cell cultures.

In order to obtain a higher fraction of soluble Si (OSA) in the Si-supplemented cell culture medium, the above protocol was modified.

As above, a 350 μg/ml Si stock was prepared by adding 100 μl of concentrated basic sodium silicate solution to 49.9 ml α-MEM (with 1% antibiotic–antimycotic and 1% L-alanyl-L-glutamine), but after through mixing and without pH-adjustment or addition of FBS, was diluted in α-MEM (supplemented with antibiotic/antimycotic and glutamine) to the desired Si concentrations (25–150 μg/ml Si) and stored at room temperature for 24 hr. Thereafter, the pH was corrected to 7.0–7.2 with HCl and the medium was stored for another 48 hr at room temperature. Finally, 10% FBS (v/v) was supplemented into the medium just before addition to the cell cultures.

2.1.2 | Analyses of Si species in cell culture medium

Aliquots of the standard preparations of the Si stock solution (350 μg/ml) and 50 μg/ml dilution were analysed for nanoparticulate silica content by dynamic light scattering (DLS). DLS measurements were performed at room temperature, with polystyrene micro-cuvettes using a Zetasizer Nano-ZS (Malvern Instruments Ltd, Malvern, UK). Since FBS interfered with the DLS measurement, the 350 μg/ml stock solution and 50 μg/ml dilution were prepared without FBS. Acquisition parameters for silicon dioxide (refractive index = 1.45, absorption = 0.01) were used, along with a 173° backscatter angle. For each sample, three replicate of 15 measurements were recorded and the average volume particle distribution reported.

Aliquots of the standard prep of the 350 μg/ml Si stock solution and 50 μg/ml dilution and the modified 50 μg/ml Si prep underwent ultrafiltration and total element analysis by inductive coupled plasma–optical emission spectroscopy (ICP–OES) to determine the fraction of soluble and particulate Si in these preparations. Briefly, 500 μl aliquots of the solutions were ultra-filtered using Vivaspin-500 centrifuge units with 3,000 MWCO membrane, at 13,000 rpm for 5 min in an Eppendorf 5804R centrifuge. The ultra-filtrate and the fraction retained by the membrane were collected and diluted with UHP water and analysed for Si content by ICP–OES as described below. To determine possible Si contamination of the sample from the ultra-filter units, four aliquots of UHP water was similar processed and analysed for Si content. Aliquots of the unfiltered Si solutions were also analysed by ICP–OES to determine total Si content.

Total elemental analysis for Si by ICP–OES was carried out on a Jobin Yvon Horiba Ultima 2C (Instrument SA, Longjumeau, France), equipped with a concentric nebuliser and cyclonic spray chamber. A sample introduction pump speed of 15 rpm, nebuliser flow rate of 0.45 L/min and a plasma gas flow rate of 10 L/min were used. Peak profiles were used as previously described,[33] with a window size of 0.075 nm (0.0375 nm either side of the peak) with 21 increments per profile and an integration time of 0.5 s per increment. The 251.611 nm analytical line for Si was used. Samples were analysed with Si standards (0–20 mg/L) prepared in diluted α-MEM, using a 1,000 mg/L Si ICP solution. Each sample/standard was analysed in triplicate and the average value used for calculations.

2.2 | Cell culturing

RAW264.7 cells (ATCC TIB-71, USA), were maintained in α-MEM supplemented with 1% antibiotic–antimycotic, 1% L-alanyl-L-glutamine and 10% (v/v) FBS at 37°C in a humidified atmosphere of 5% CO_2. Cell passages between three and seven were used in the experiments described herein. Mycoplasma tests were performed regularly in order to control for contamination.

For experiments, cells were seeded into 24 or 96 well plates (Nunc, ThermoFisher, Waltham, MA) with the α-MEM culture media. After 24 hr, to allow for cell attachment, non-adherent cells were removed and adherent cells incubated with the Si-supplemented medium (prepared as described in Section 2.1) for 48 or 96 hr, with medium change every 48 hr. RANKL (5 ng/ml; E-coli expressed recombinant mouse RANKL, R&D Systems, Minneapolis, MN) was added to the cell culture medium when investigating osteoclast differentiation.

2.3 | Effect of Si on cell viability using the neutral red assay

The neutral red (NR) uptake assay, as described by Repetto et al.,[34] was used to assess the effects of Si on cell viability. RAW264.7 cells were cultured in 96-well plates (10^4 cells per well) for 24 hr with standard or modified preparations of the Si-supplemented cell culture medium (0–150 μg/ml Si) and with or without RANKL (5 ng/ml). NR incubation medium (40 μg/ml) was prepared by dissolving the NR dye (Merck, Germany) in phosphate buffered saline (PBS; Gibco, ThermoFisher, Waltham, MA) to 4 mg/ml and then diluted 1:100 in α-MEM. The NR incubation medium was incubated at 37°C for 24 hr and then centrifuged for 10 min at 600 × g to remove precipitated dye crystals. Cells were incubated with NR medium (100 μl per well) at 37°C for 2 hr, then washed with PBS (150 μl per well). NR destain solution (50% EtOH-96%, 49% deionised water and 1% acetic acid) was added (150 μl per well) and the plates were shaken for 10 min to release the NR dye from the cell lysosomes. The amount of viable cells was quantified by measuring the absorbance of the released NR dye at 540 nm on an optical microplate reader (Multiskan FC, ThermoScientific, Waltham, MA). The experiment was repeated three times with eight replicates per treatment.

2.4 | Effect of Si on osteoclastogenesis

RAW264.7 cells were cultured in 24-well plates (5×10^3 cells per well) with or without RANKL (5 ng/ml) for 48/96 hr. Silicon (50 μg/ml) was also present either for the entire 48/96 hr period or for shorter durations (0–24, 0–48, and 0–72 hr) or added at different 24 hr intervals over the 96 hr (24–48, 48–72, and 72–96 hr). Following incubation with Si-supplemented media, cells were washed twice with PBS (500 μl per well) and cultured for the remaining period in α-MEM with RANKL (5 ng/ml). Cells were then stained for TRAP to identify

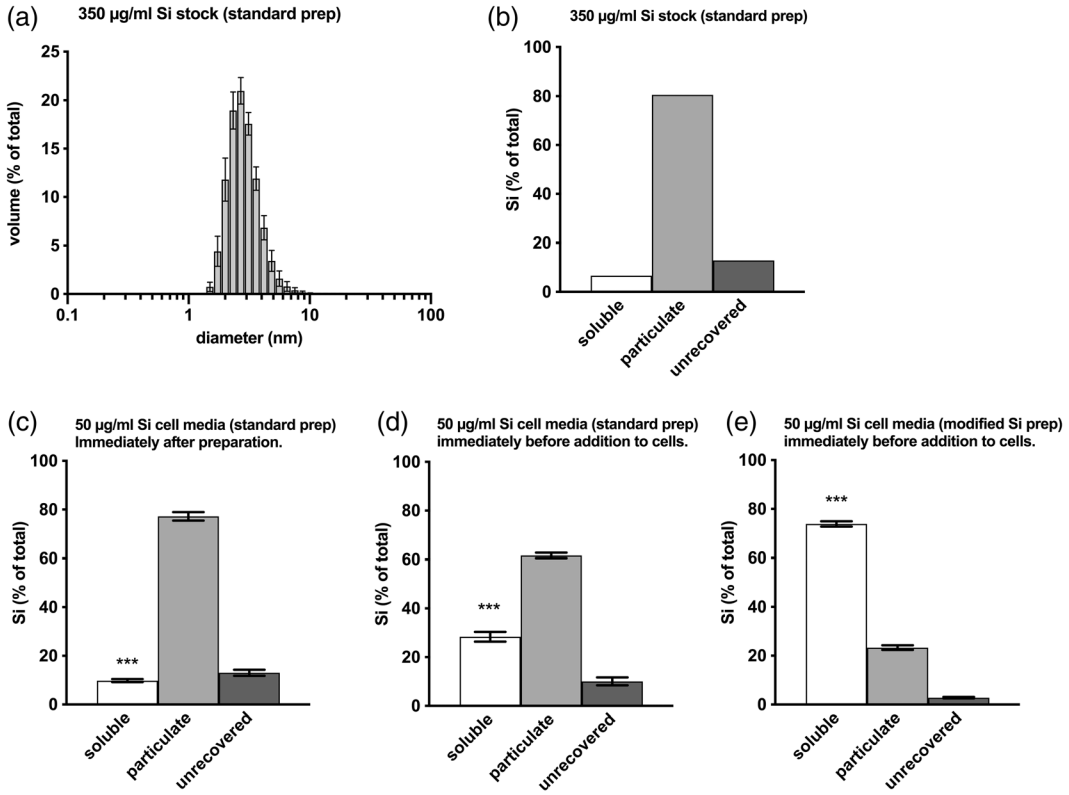

FIGURE 1 Soluble and particulate Si in the Si-supplemented cell culture medium. (a) Particle size distribution by dynamic light scattering and (b) ultrafiltration analysis of the standard preparation of the 350 µg/ml Si stock immediately after preparation and at the point of dilution to lower Si concentrations in cell culture medium. The fraction of ultrafilterable (<3 kDa, soluble)□, recovered (>3 kDa, particulate)▒ and unrecovered Si■, are shown. Ultrafiltration analysis of the 50 µg/ml Si-supplemented cell culture medium immediately after preparation from the 350 µg/ml stock (c) and after 24 hr standing and at the point of addition to the cells (d). Ultrafiltration analysis of the modified preparation of the 50 µg/ml Si-supplemented medium, at the point of addition to cells (e). Silicon supplemented cell culture medium were prepared as described in Materials and Methods. Data are presented as mean ± SEM of at least three independent preparations ($N = 3$) except for 350 µg/ml Si stock (one preparation analysed in triplicates by ultrafiltration). Significant differences in percentage ultrafilterable Si between the two different 50 µg/ml Si preps and between the different times points after prep are shown: ($p < .001$)***

multinucleated osteoclast-like cells or the RNA extracted and assessed by RT-qPCR for phenotypic markers of osteoclast differentiation (see below). The experiment was repeated three times with four replicates per treatment.

2.4.1 | TRAP-staining of osteoclast-like cells

After 96 hr and after washing with PBS (2 × 500 µl per well), cells were fixed with citrate-acetone (500 µl per well with a solution containing 27% 27 mM Citrate solution and 73% of a 2.5 M Acetate solution) for 30 s and air-dried at room temperature. Cells were stained for tartrate-resistant acid phosphatase (TRAP) using a commercially available kit (Acid Phosphatase Leukocyte staining kit, Sigma-Aldrich, St. Louis, MO) and following the manufacturer's protocol but with a reduced staining time of 20 min. Cells stained dark red with three or more nuclei were counted as osteoclasts. Images were captured with a Zeiss Primo Vert light microscope under ×5 magnification.

2.4.2 | Relative quantification of gene expression analysis by RT-qPCR

The minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines checklist was followed to ensure the relevant experimental conditions and assay characteristics.[35]

Total RNA was extracted from the cell lysate with the RNeasy Mini Kit (Qiagen, Germany) according to manufacturer's protocol. Briefly, cell culture medium was removed and cells were washed with PBS, then lysed and homogenised. The cell lysates were centrifuged through a gDNA eliminator spin column. Ethanol (70%) were then added to the flow through making the RNA bind to the membrane in the spin column and the columns were repeatedly rinsed and washed with several buffers. The purified RNA were eluted in 80 µl of RNase free water. RNA concentration was quantified with the Qubit RNA broad range assay kit and Qubit fluorometer (Invitrogen, ThermoFisher, Waltham, MA). RNA quality was assessed using RNA StdSens Analysis chip by Experion (Bio-Rad Laboratories, Hercules, CA). RNA were kept at −80°C until reverse

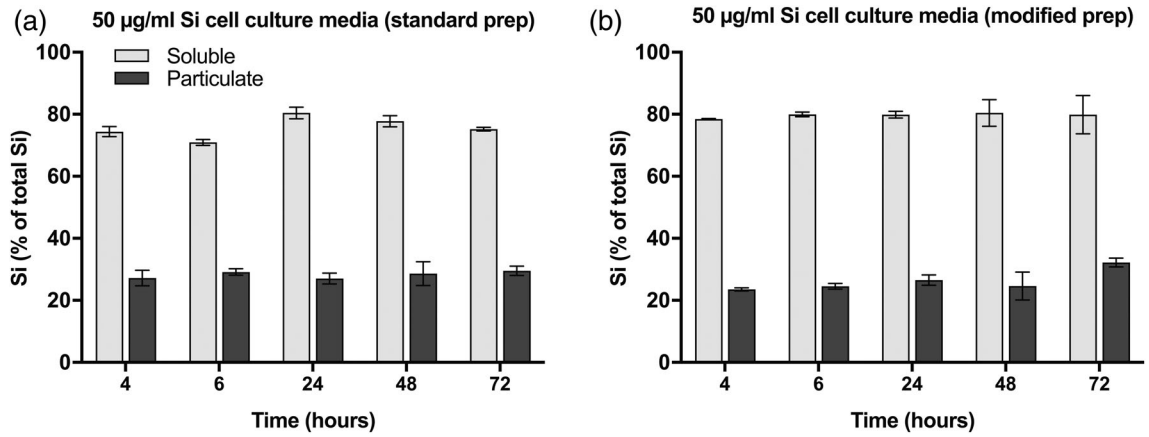

FIGURE 2 Soluble and particulate Si in the 50 µg/ml Si-supplemented cell culture medium under cell incubating conditions (37°C and 5% CO_2). Ultrafiltration analysis of the (a) standard (partly soluble) and (b) modified (highly soluble) preparations of the 50 µg/ml Si-supplemented media following incubation under cell culture conditions for 4–72 hr. The fraction of ultrafilterable (<3 kDa, soluble) and recovered (>3 kDa, particulate) Si is shown. Data is mean ± SEM of three independent preparations of the 50 µg/ml Si-supplemented cell culture medium (N = 3)

transcription (RT). RT was performed on a MiniOpticon (Bio-Rad Laboratories, Hercules, CA) using iScript gDNA Clear cDNA Synthesis kit (Bio-Rad Laboratories, USA) with 0.1 µg total RNA in 20 µl reaction volumes. Identification of any possible inhibition in the RT and the subsequent RT-qPCR assay, was controlled by adding 0.01 ng of Universal RNA Spike template (TATAA Biocenter, Sweden) to each RT reaction.

To compensate for possible variation in the amount of genetic material, the target gene expression was normalised towards a selected reference gene with a constant and non-regulated expression. The most stable reference gene for RAW264.7 cells was selected by screening 12 potential reference genes (mouse Reference gene panel, TATAA Biocenter, Sweden) on the different samples in the experiment.[36] Analysis of the expressions by geNorm and Normfinder algorithms (GenEx software, Multid Analyses, Sweden) showed that the most stable reference genes were peptidylpropyl isomerase A (*PPIA*) and TATA-box binding protein (*TBP*).

Gene expression analysis was assessed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and wet lab validated predesigned primers (Bio-Rad Laboratories, Hercules, CA). The primer assay identification numbers were Receptor Activator of Nuclear Factor K-B (*RANK*) (qMmuCED0046133), Nuclear Factor of Activated T-cell 1 (*NFATc1*) (qMmuCID0040129), Dendritic Cell Specific Transmembrane Protein (*DC-STAMP*) (qMmuCID0019866), Calcitonin Receptor (*CalcR*) (qMmuCID0021168), Cathepsin K (*CtsK*) (qMmuCID0022824), Tartrate Resistant Acid Phosphatase (*TRAP*) (qMmuCED0044793), TATA-box Binding Protein (*TBP*) (qMmuCID0040542), and Peptidylprolyl Isomeras A (*PPIA*) (qMmuCED0041303). The expression of the target genes and the two reference genes were analysed with 1 ng cDNA in a 10 µl reaction volume in duplicates on a CFX Connect Real-Time System with the CFX manager software (Bio-Rad Laboratories, Hercules, CA). As all the samples did not fit in one PCR plate layout, an interplate calibrator (TATAA Biocenter, Sweden) was added in triplicates to each PCR plate to compensate for variations between qPCR runs. Melting curves for each reaction were performed between 60 and 95°C to detect any primer dimers. The qPCR results were normalised to *PPIA* and *TBP* and compared to positive control (RANKL). As all assays had 90–100% efficiency, $2^{-\Delta\Delta Cq}$ was used to calculate the relative gene expression.

2.5 | Statistics

Results are reported as means ± SEM unless otherwise stated. Normal distribution of the data was confirmed with the Shapiro–Wilk normality test. One-way ANOVA followed by Tukey's multiple comparisons test was used to compare between treatments and significance was taken when $p < .05$. All statistical analyses were performed in GraphPad PRISM 7 (GraphPad Software). In the figures, statistical significance was denoted as: $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$.

3 | RESULTS

3.1 | Characterisation of Si-supplemented cell culture medium

Standard preparation of Si-supplemented cell culture media were prepared as previously described[22] and Si concentrations of the 350 µg Si/ml stock solution and 50 µg/ml dilution were verified by ICP–OES; mean values were 375 ± 12.6 (SD) and 52.5 ± 1.2 (SD) µg Si/ml respectively ($N \geq 3$). Particle sizing, by DLS, of the 350 µg Si/ml stock immediately after preparation and at the time of dilution to lower Si concentrations, showed the presence of particles between 1.5 and 7.5 nm in diameter (median particle diameter of 2.7 nm, Figure 1a).

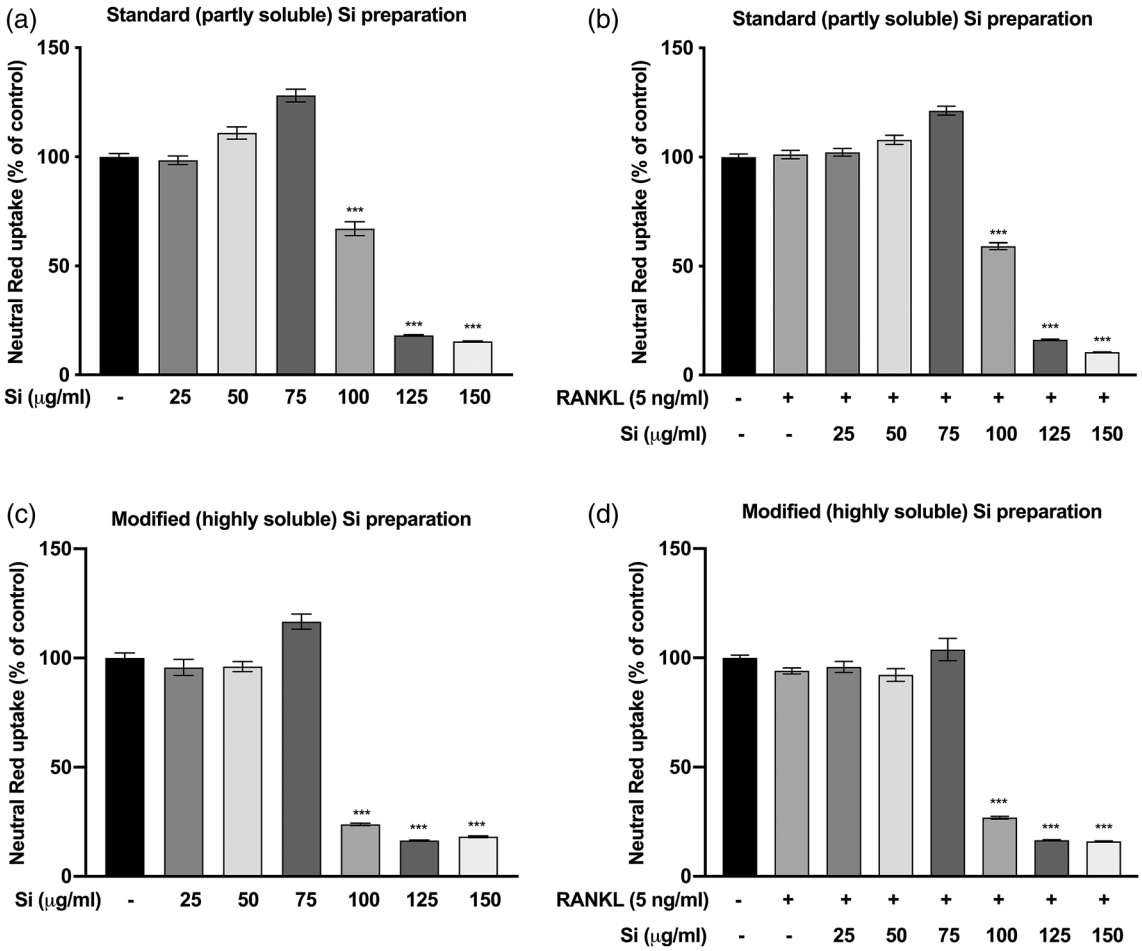

FIGURE 3 Effects of Si on Neutral Red uptake in RAW264.7 cells. Neutral Red uptake was used to assess cell viability/proliferation in RAW264.7 cells 24 hr after culturing with either standard (partly soluble) (a and b) or modified (highly soluble) (c and d) preparations of the Si-supplemented cell culture medium and with (b and d) or without (a and c) RANKL (5 ng/ml). Silicon concentration span 0–150 μg/ml. Data are presented as mean percentage of control ± SEM of eight samples from a representative experiment (N = 8), with the control set to 100%. Significant differences compared to control are shown: (p < .001)***

TEM, ^{29}Si-NMR and zeta potential analyses of similar size Si particles, prepared from the same source (alkaline sodium silicate) and the same methodology/protocol has been previously described by us.[37,38] Note the 350 μg Si/ml stock is meta-stable since the particles doubled in size with every hour of standing after preparation (Figure S1). Particle sizing could not be carried out on the 50 μg/ml Si-supplemented media due to the low particle count. Therefore, ultrafiltration studies were performed to determine the fractions of soluble (ultrafilterable, < 3,000 MWCO) and particulate Si. The 350 μg Si/ml stock solution, immediately after preparation and at the time of dilution to lower Si concentrations, contained 7% ultrafilterable (soluble) Si (Figure 1b). The 50 μg/ml Si-supplemented media similarly contained 10% ultrafilterable Si immediately after preparation from the 350 μg/ml stock (Figure 1c), and on standing for 24 hr and just prior addition to cells, the amount of ultrafilterable Si in 50 μg/ml Si-supplemented media increased to 28% (Figure 1d; p < .001). To increase the proportion of ultrafilterable (soluble) Si in the Si-supplemented media, the method of preparation was modified, as described in Methods. Ultrafiltration study showed that the modified preparation of the 50 μg/ml Si-supplemented media contained 74% ultrafilterable Si at the time of addition to the cells (Figure 1e), significantly more than in the standard preparation (p < .001). To distinguish between the two different preparations of the Si-supplemented media and their content of soluble Si, we use the term "Standard (partly soluble) Si prep" and "Modified (highly soluble) Si prep" in the text.

To investigate how the proportions of ultrafilterable (soluble) Si changes in the 50 μg/ml Si-supplemented media under cell culturing conditions, that is, 37°C and 5% CO_2, further ultrafiltration analysis were performed. Aliquots of the 50 μg/ml Si-supplemented media were incubated at 37°C and 5% CO_2 for different durations (4–72 hr) prior to ultrafiltration analysis. As shown in Figure 2, after 4 hr of incubation under cell culture conditions the proportion of ultrafilterable (soluble) Si in the standard (partly soluble) prep of the 50 μg/ml Si-supplemented media had increased to 74% (Figure 2a), markedly more than at the time of addition to the cells (Figure 1d), and maintained this level of soluble Si for the entire 72 hr. These levels of soluble Si were slightly lower, but comparable to that seen in the modified (highly soluble) prep of the 50 μg/ml Si-

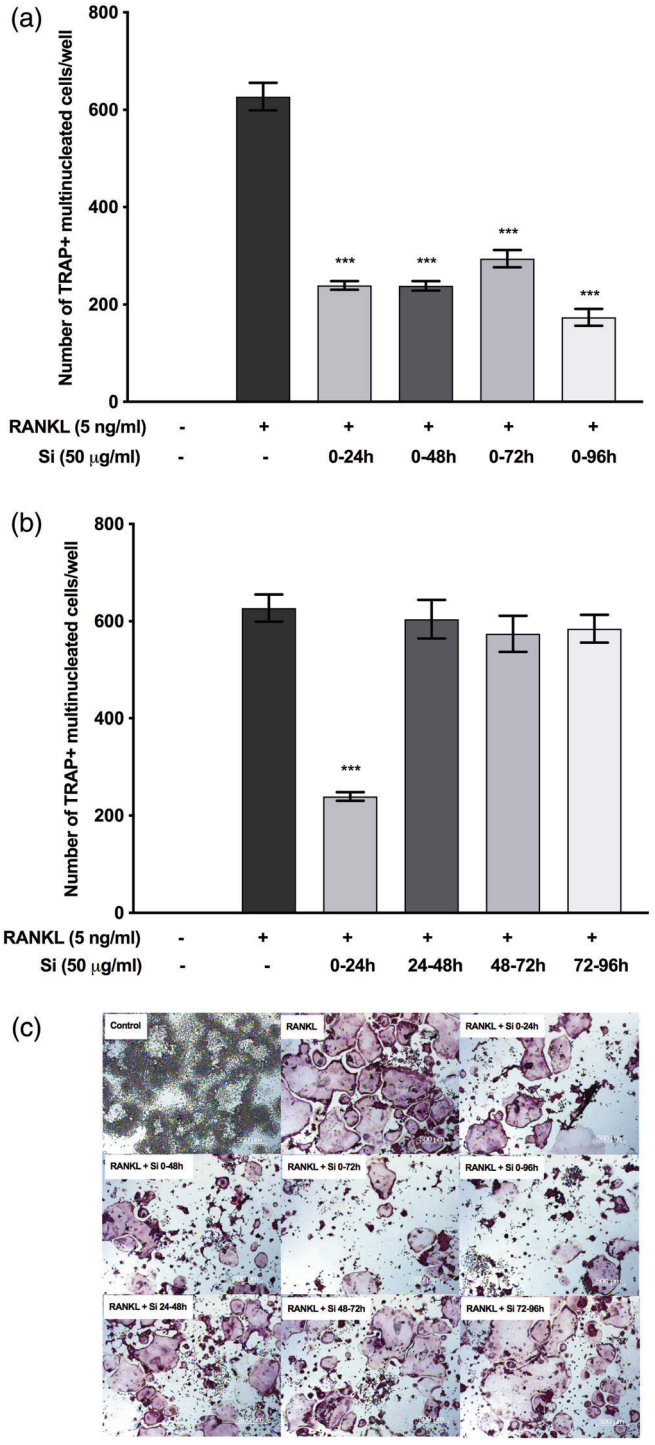

FIGURE 4 Effects of Si on osteoclast formation in RAW264.7 cells. RAW264.7 cells was cultured with RANKL (5 ng/ml) and with the standard (partly soluble) Si preparation of the 50 μg/ml Si-supplemented cell culture medium, at different time intervals and 24 hr durations over a 96 hr cell culture period. Silicon was present for (a) increasing 24 hr durations, 0–24 hr and up to 96 hr or (b) added at different 24 hr time intervals over the 96 hr. The number of TRAP+ multinucleated cells are presented as mean ± SEM of four samples from a representative experiment ($N = 4$). Significant differences compared to RANKL alone are shown: ($p < .001$)***. (c) Representative micrographs showing cell morphology and distribution in the corresponding treatment groups after 96 hr culture

supplemented media investigated under the same conditions (Figure 2b).

3.2 | Effects of Si on neutral red uptake

Neutral Red (NR) uptake by RAW264.7 cells was assessed after 24 hr incubation with Si (0–150 μg/ml) in the presence and absence of RANKL (5 ng/ml). RAW264.7 cells treated with either the standard (partly soluble) or modified (highly soluble) prep of the Si-supplemented cell culture medium showed significantly reduced uptake of NR at Si concentrations ≥100 μg/ml ($p < .001$, Figure 3). The 100 μg/ml modified (highly soluble) Si prep showed a more pronounced reduction in NR uptake than the standard (partly soluble) Si prep. With 75 μg/ml Si, a slight increase in NR uptake was observed with both the standard (partly soluble) and modified (highly soluble) Si preps, whilst with 50 μg/ml Si, there was no difference in NR uptake, compared with control, with either Si preps. The 50 μg/ml Si concentration, showing no effect on cell viability/proliferation, was therefore chosen for the further experiments described below. RANKL had no influence on the observed Si effects.

3.3 | Effects of Si on osteoclast formation

The effects of the standard (partly soluble) prep of the 50 μg/ml Si-supplemented medium, added at different periods during osteoclast formation was investigated (Figure 4). These experiments were carried out in the presence of RANKL as TRAP+ multinucleated cells (differentiated/mature osteoclasts) are formed only in RANKL-stimulated cultures. Washing with PBS (i.e., following Si incubation) did not have any effects on the number of TRAP+ multinucleated cells (data not shown). The number of TRAP+ multinucleated cells was significantly reduced compared with RANKL-stimulated cells ($p < .001$), when 50 μg/ml Si was present for at least the first 24 hr of the 96 hr culture period (Figure 4a,c). No further reduction in TRAP+ cells was seen with longer periods of incubation with 50 μg/ml Si (i.e., > 0–24 hr, Figure 4a,c). In contrast, when 50 μg/ml Si was added at any time after the first 24 hr, no reduction in the number of TRAP+ cells was seen (Figure 4b,c).

3.4 | Effects of Si on gene expression

The relative expression of *RANK*, *NFATc1*, *DC-STAMP*, *TRAP*, *CtsK*, and *CalcR* in RAW264.7 cells, following incubation with or without RANKL (for 48 or 96 hr) and with or without standard (partly soluble) prep of the 50 μg/ml Si-supplemented medium for 0–24, 0–48, or 0–96 hr, was assessed by RT-qPCR (Figure 5). *RANK*, *NFATc1*, and *DC-STAMP* expression were detected in both control and RANKL-stimulated cells (Figure 5a–f), with significantly highly ($p < .001$) expression of *NFATc1* and *DC-STAMP* in RANKL-stimulated cells (Figure 5c–f). *RANK*

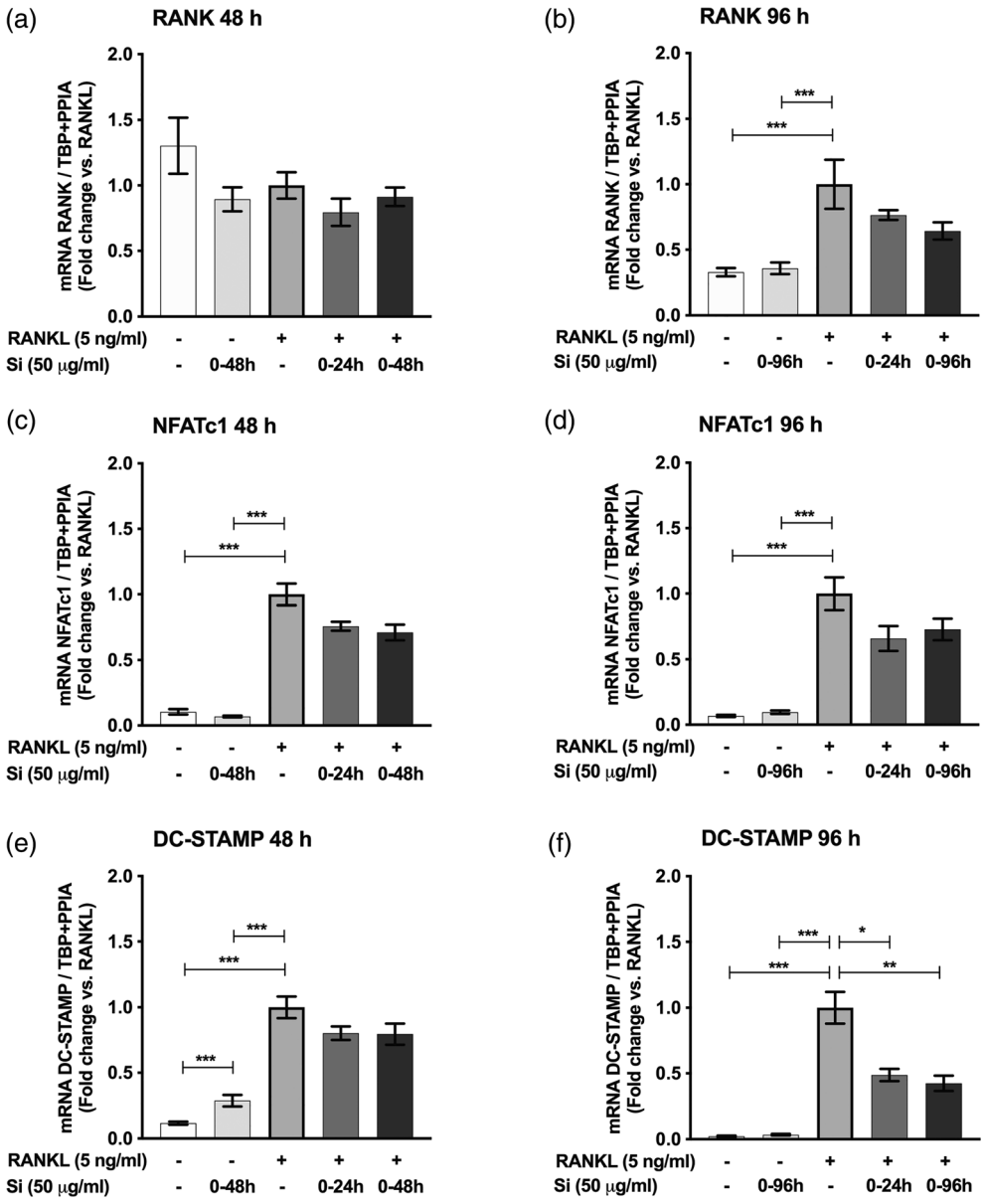

FIGURE 5 Effects of Si on gene expressions in RAW264.7 cells. Expression of *RANK*, *NFATc1*, *DC-STAMP*, *CalcR*, *CtsK*, and *TRAP* genes in RAW264.7 cells cultured with the standard (partly soluble) preparation of the 50 μg/ml Si-supplemented cell culture medium for 48 hr (a, c, e, g, i, and k) or 96 hr (b, d, f, h, j, and l), with or without RANKL (5 ng/ml). Gene expressions are normalised to *TBP* and *PPIA*. Values are presented as mean fold changes relative to RANKL ± SEM of four samples from two representative experiment (N = 6–8). Significant differences are shown: (p < .05)*, (p < .01)**, (p < .001)***

expression was also significantly higher (p < .001) in RANKL-stimulated cells (compared to unstimulated cells), but only in the 96 hr cultures (Figure 5b). *CalcR*, *CtsK*, and *TRAP* were only expressed in RANKL-stimulated cells (Figure 5g–l). Silicon (50 μg/ml) did not significantly alter the expression of *RANK* and *NFATc1* in either control or RANKL-stimulated RAW264.7 cells (Figure 5a–d). However, *CtsK* and *TRAP* expression were significantly inhibited (p < .001) when Si was present for the entire culturing period (0–48 / 0–96 hr; Figure 5i–l). Silicon (50 μg/ml) also significantly inhibited the expression of *DC-STAMP* (p = .002) and *CalcR* (p < .001) in the RANKL-stimulated cells, but only in 96 hr culture (Figure 5f,h). Furthermore, exposure of the cells to Si over the first 24 hr of culture was sufficient to cause significant inhibition of *CtsK* (p = .039), *TRAP* (p = .002), *DC-STAMP* (p = .018), and *CalcR* (p < .001) in the 96 hr culture (0–24 hr; Figure 5f,h,j,l).

3.5 | Comparison of the standard (partly soluble) and modified (highly soluble) preparations of the Si-supplemented cell culture medium on gene expression

CtsK, *TRAP*, and *CalcR* expression, in RANKL-stimulated RAW264.7 cells, were then compared between the standard (partly soluble) and modified (highly soluble) Si prep of the 50 μg/ml Si-supplemented media. The modified (highly soluble) prep of the 50 μg/ml Si-

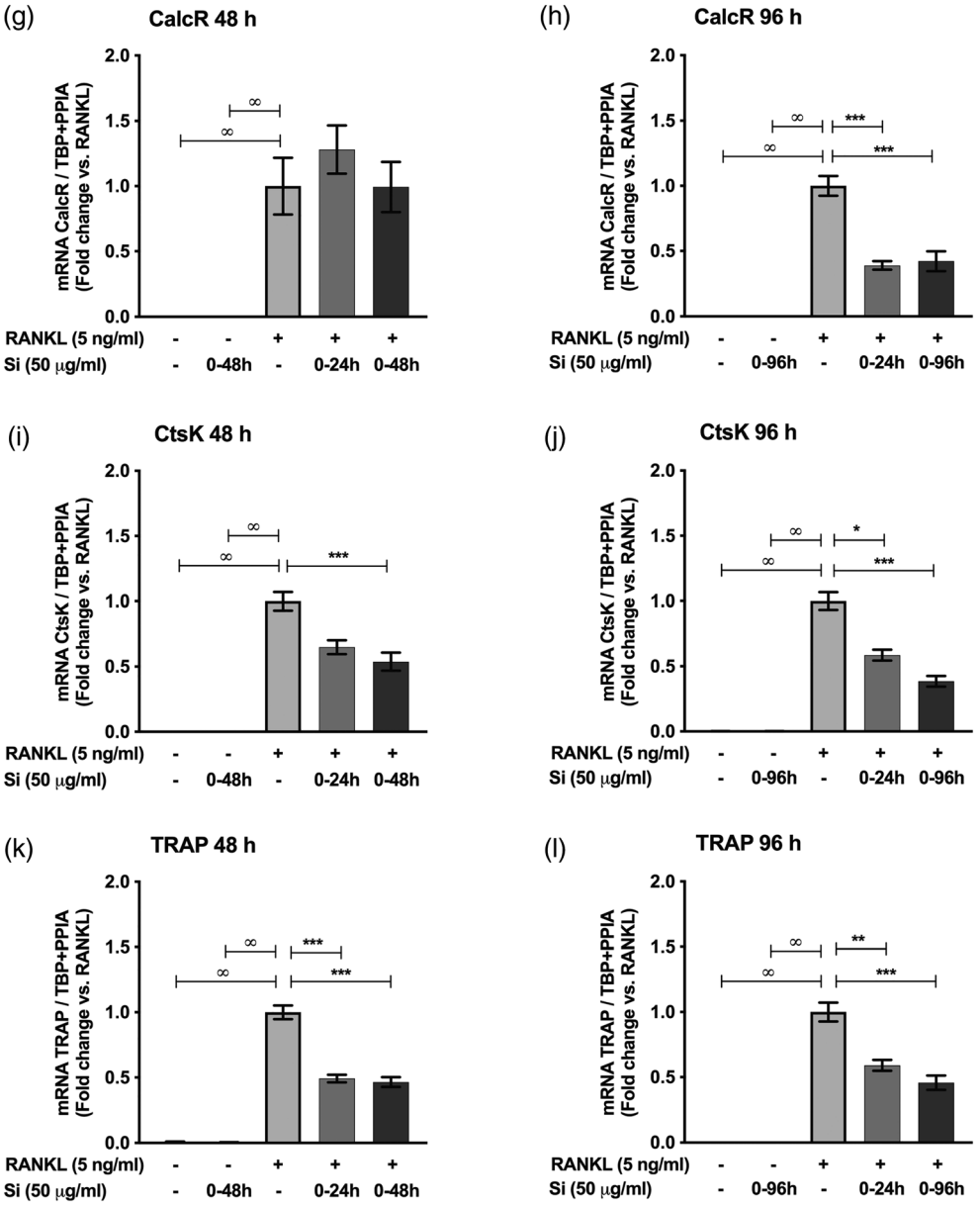

FIGURE 5 (Continued)

supplemented cell culture medium significantly inhibited *CtsK* expression (p = .041), to the same extent as the standard (partly soluble) Si prep (p = .019; Figure 6a). Similarly, *TRAP* and *CalcR* expressions were also significantly supressed, by the same extent, with both the modified (highly soluble) and standard (partly soluble) preparation of the 50 μg/ml Si-supplemented media (p < .001; Figure 6b,c). There was no significant difference in the extent of inhibition of *CtsK*, *TRAP*, and *CalcR* expression between the modified (highly soluble) and standard (partly soluble) preparations of the 50 μg/ml Si-supplemented media.

4 | DISCUSSION

Silicon is a constituent of the human body, at comparable level to iron and zinc, but very little is known about its physiological role and therefore its clinical importance.[14,39] Silicon is present in all tissues of the rat and highest levels are found in bone and other connective tissues.[40,41] Levels in human bone and connective tissues have not been established, although similar levels to rats are expected. The bioavailable form of Si in the diet is the soluble OSA specie and the same species is found in human blood. The Si specie that acts on and accumulates in bone and other connective tissues has not been established, but modelling studies suggest that, at least in bone, it is likely to be OSA, in a deprotonated form.[42] Soluble Si (OSA) is therefore considered as the biologically relevant Si specie. OSA is the predominant form of Si in aqueous solutions at neutral pH and at Si concentrations <56 μg/ml (2 mM).[11] At concentrations >56 μg/ml, OSA starts to polymerise (auto condense) to form polymers that have reduced solubility and hence bioavailability.[11] Both the standard (partly soluble) and modified (highly soluble) preparations of the

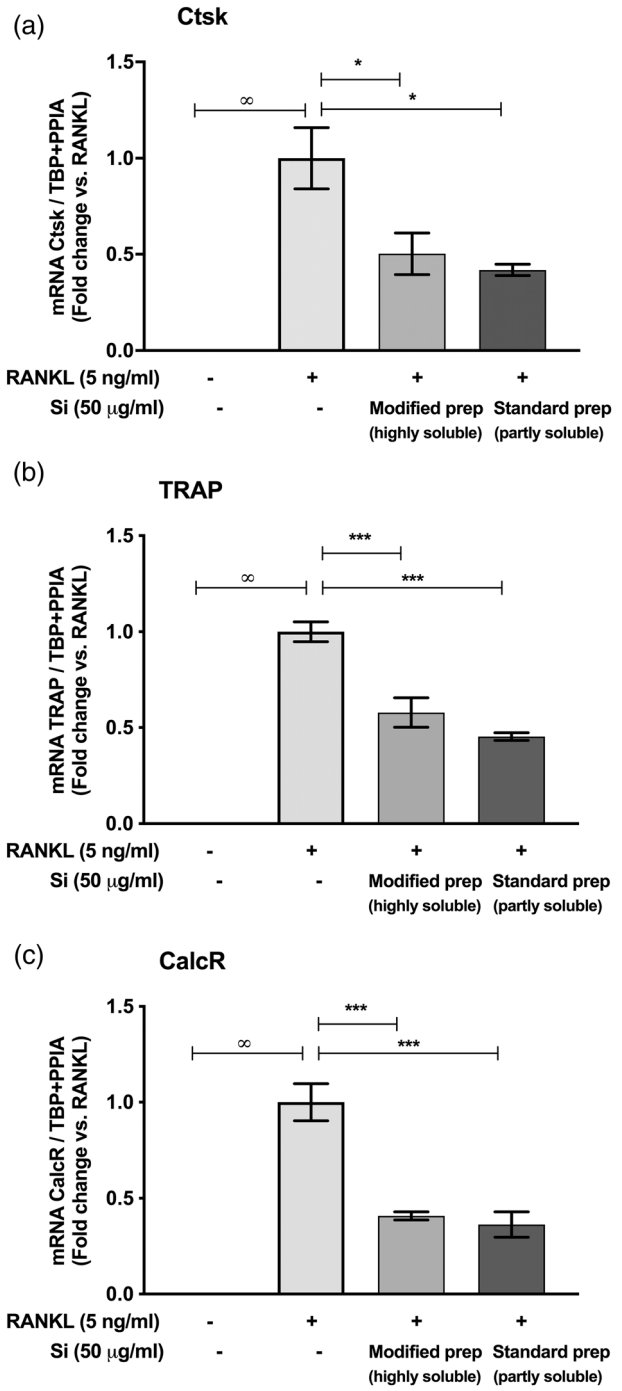

FIGURE 6 Comparison of the standard and modified Si preparations on gene expressions in RAW264.7 cells. Expression of *Cathepsin K*, *TRAP* and *Calcitonin receptor* genes by RAW264.7 cells after 96 hr culture in either the standard (partly soluble) or modified (highly soluble) preparations of the 50 μg/ml Si-supplemented cell culture medium, in the presence of RANKL (5 ng/ml). Gene expression are normalised to *TBP* and *PPIA* and presented as mean fold changes relative to RANKL ± SEM of four samples from a representative experiment ($N = 4$). Significant differences compared to RANKL are shown: ($p < .05$)*, ($p < .001$)***

50 μg/ml Si-supplemented cell culture medium, used in the present study, are in the pH and concentration range for OSA to be the dominant specie. However, the proportion of OSA at time of addition to the cells differed between the two solutions and was dependent on the method of preparation. The modified (highly soluble) preparation contained significantly higher levels of OSA at the time of addition to the cells. Serum, which is present in the cell culture medium contains proteins that may aggregate Si and/or slow down the dissolution of particulate Si to OSA. Thus, on dilution (to below 56 μg/ml Si) the solution will require a longer period of incubation for equilibrium between particulate and soluble Si to be reached. In the modified (highly soluble) Si preparation, serum was only added after dilution to 50 μg/ml and only after allowing time for any particulate Si, present in the 350 μg/ml stock, to dissolve. Additionally, the pH (∼9.5–10) of the 350 μg/ml Si stock used for the modified (highly soluble) Si prep was not neutralised, nor was serum added, again to limit particulate Si formation, in contrast to the standard (partly soluble) prep. However, following incubation of the standard (partly soluble) 50 μg/ml Si-supplemented media at 37°C and 5% CO_2, to mimic the cell culture conditions, a marked increase in the proportion soluble Si was observed. The levels of soluble Si was comparable to that seen in the modified (highly soluble) prep of the 50 μg/ml Si-supplemented media. This implies that cell culture conditions accelerates the dissolution of the particles in the standard (partly soluble) prep of the 50 μg/ml Si-supplemented media and equilibrium is attained earlier. These findings can explain the comparable cellular results obtained with the two different 50 μg/ml Si preparations. In contrast to the present study, most reports on the biological effects of Si in vitro present no speciation of the Si culture medium and therefore the active Si specie in these reports is either not known or implied to be OSA.

Some reports suggest that human skin epithelial cells and T cells can readily take up small amorphous silica polymers (nanoparticles) in the culture medium,[38,43] which then undergoes intracellular dissolution releasing OSA at a faster rate than uptake of OSA from the cell medium.[43] Similar effects of silica nanoparticles on osteoblast cells have been reported.[29] It is not known if RAW264.7 cells are capable of phagocytosing silica particles and if this could contribute to the inhibitory effect of OSA on osteoclastogenesis. However, the fact that the modified prep, which contained predominantly soluble Si with very little particulate Si, produced the same effects as the standard (partly soluble) prep of the Si-supplemented cell culture media, strongly suggest that OSA is the biologically active Si specie and the ultrafiltration studies conducted here provides the evidence for this conclusion.

In accordance with our previous study, 50 μg/ml Si did not affect the viability of RAW264.7 cells but reduced the number of TRAP+ osteoclast-like cells in the RANKL-stimulated cultures.[22] This confirmed that Si supresses osteoclastogenesis. Here we investigated at what stage(s) of osteoclast differentiation Si acts upon. Osteoclastogenesis consists of several steps, including differentiation and fusion of mononuclear precursor cells into multinucleated osteoclasts.[44-47] Interestingly, the presence of Si during the first 24 hr of the RANKL-stimulated cultures reduced the number of multinucleated TRAP+ cells to the same extent as when Si was present for the entire 96 hr incubation period. In contrast, when Si was present after the first 24 hr period, no suppression in TRAP+ cells was seen. This

strongly suggests that Si acts upon the initial regulatory steps in osteoclastogenesis. To investigate this further, the effects of Si on gene expression of some characteristic osteoclast markers was assessed. In RAW264.7 cells, osteoclastogenesis is initiated by the binding of RANKL to the RANK receptor.[45] The activation of RANK leads to a signalling cascade that activates crucial transcription factors such as *NFATc1*, which in turn induces the expression of several osteoclast specific genes.[48] In our study, the expression of both *RANK* and *NFATc1* were unaffected by Si, but *DC-STAMP*, *CalcR*, *CtsK*, and *TRAP* were clearly inhibited. DC-STAMP is a transmembrane protein that is essential for the fusion of osteoclast progenitors.[49] In DC-STAMP$^{-/-}$ mice, multinucleation is abolished as shown by the lack of TRAP+ multinucleated cells.[49] Similarly, in the present study, the expression of both *DC-STAMP* and *TRAP* were suppressed and a decrease in the number of multinucleated TRAP+ cells was seen in the Si treated cultures. Interestingly, Si exposure for just the first 24 hr supressed *DC-STAMP* expression to the same extent as Si exposure over the entire 96 hr. The terminal differentiated osteoclast is characterised by the expression of phenotypic markers such as *CalcR*, *CtsK*, and *TRAP*.[30] Here, in addition to a decrease in the number of TRAP+ multinucleated cells, the expression of *CalcR*, *CtsK*, and *TRAP* were also significantly inhibited. The standard (partly soluble) and modified (highly soluble) 50 μg/ml Si preparations inhibited these genes by comparable amounts. Our study clearly shows that Si may have a direct inhibitory effect on osteoclastogenesis that does not require mediation via stromal/osteoblast cells.

In conclusion, the results from the present study confirms the direct inhibitory effects of Si on osteoclastogenesis. Our findings suggest that inhibition by Si occurs in the early stages of osteoclast differentiation. Moreover, we can confidently attribute these findings to the biologically relevant soluble Si specie (OSA).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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