Title: Extracellular Histone Promotes Calcium Phosphate-Dependent Calcification in Mouse Vascular Smooth Muscle Cells

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Running title: **Extracellular Histone Drives Vascular Calcification in VSMCs**

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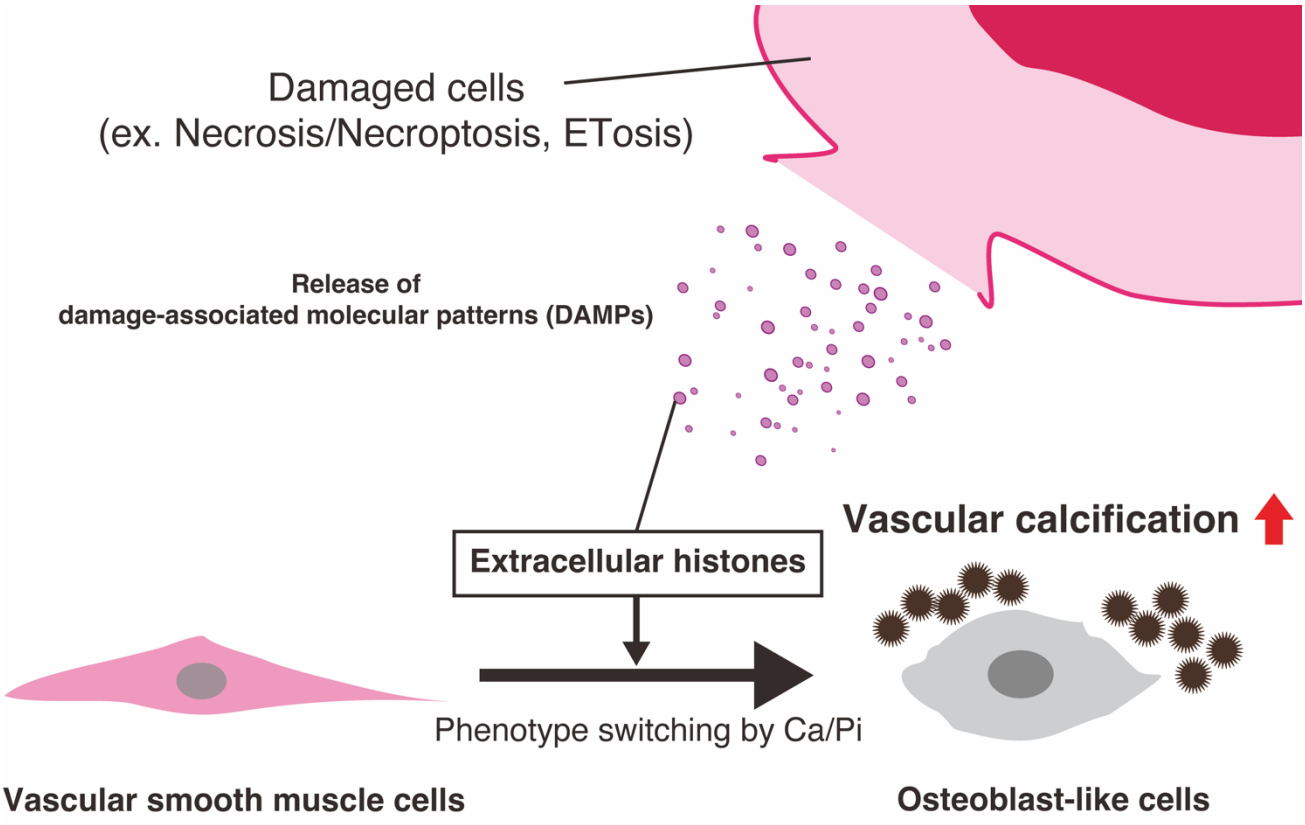
Graphical abstract



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Summary

Vascular calcification, a major risk factor for cardiovascular events, is associated with a poor prognosis in chronic kidney disease (CKD) patients. This process is often associated with the transformation of vascular smooth muscle cells (VSMCs) into cells with osteoblast-like characteristics. Damage-associated molecular patterns (DAMPs), such as extracellular histones released from damaged or dying cells, are suspected to accumulate at calcification sites. To investigate the potential involvement of DAMPs in vascular calcification, we assessed the impact of externally added histones (extracellular histones) on calcification within mouse VSMCs. These cells included MOVAS cells and primary VSMCs, which were exposed to high levels of calcium and inorganic phosphate (Pi). Our study found that extracellular histones intensified calcification in the VSMCs. We also consistently observed a decrease in the expression of *Tagln*, a VSMC marker gene, along with an increase in the osteoblast marker gene, *Spp1*, in the MOVAS cells. However, the addition of extracellular histones did not influence cell death within the MOVAS cells. Our findings suggest that extracellular histones might contribute to calcium and phosphate (Ca/Pi)-dependent calcification in VSMCs, which operate independently of cell death. Therefore, extracellular histones could play a pivotal role in the vascular calcification observed in CKD.

Keywords: chronic kidney disease (CKD), damage-associated molecular patterns (DAMPs), extracellular histones, vascular calcification, vascular smooth muscle cells (VSMCs)

Abbreviations

ANOVA: analysis of variance, CKD: chronic kidney disease, DAMPs: damage-associated molecular patterns, DMEM: Dulbecco's Modified Eagle's Medium, FBS: fetal bovine serum, Pi: inorganic phosphate, SEM: standard error of the mean, VSMCs: vascular smooth muscle cells.

Introduction

In patients with chronic kidney disease (CKD), vascular calcification is a significant risk factor for mortality [1]. End-stage CKD patients often have abnormal deposits of calcium in the aorta and artery (known as vascular calcification), in correlation with a phenotype switch from vascular smooth muscle cells (VMSCs) to osteogenic cells. Multiple studies have shown that the dysregulation of calcium and phosphate metabolism may be involved in the osteogenic switch of VSMCs [2], but the exact mechanism of an osteogenic switch in VSMCs is not fully understood. In addition, no treatment for vascular calcification in CKD has yet been developed.

Damage-associated molecular patterns (DAMPs), endogenous molecules released by damaged or dying cells, participate in various diseases such as cancer, neurodegenerative disease, and autoimmune disease [3]. DAMPs include a variety of intracellular components, such as histones, high mobility group box 1, heat shock proteins, and DNA (nuclear and mitochondrial) [3], and evoke sterile inflammation signaling via various receptors such as Toll-like receptors, C-type lectin receptors, NOD-like receptors, RIG-I-like receptors, and cytosolic DNA sensors [3,4].

In the aorta of CKD patients, immune cells migrate and accumulate, potentially indicating DAMPs accumulation in the lesion site [5]. Extracellular histones, as DAMPs, influence inflammation through the activation of various receptors (TLR4, TLR9, Aim2, Clec2d) and their subsequent downstream pathways [3,4,6]. However, it remains unknown whether extracellular histones participate in vascular calcification. In this study, we evaluated the effect of extracellular histones on vascular calcification in mouse VSMCs.

Materials and Methods

Cell culture

Mouse VSMC cell line MOVAS (ATCC, #CRL-2797, RRID:CVCL_0F08) was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Wako, Osaka, JPN, #043-30085) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA, cat#173012), 100 U/mL penicillin (Sigma-Aldrich, #P3032) and 100 μg/mL streptomycin (Sigma-Aldrich, #S9137).

MOVAS cells were seeded at 2,000 cells/well in a 48-well plate (Corning, Corning, NY, USA, #353078). When they reached confluence on day 3, the cells were exposed to fresh medium supplemented with 1.8 mM $CaCl_2$ (Wako, #031-00435) and 2.9 mM inorganic phosphate (Pi; NaH_2PO_4/Na_2HPO_4, pH7.4) (calcification medium) with or without histones and cultured for additional 7 days. Sterilized distilled water was used as a control. We used calf thymus-derived H2A histones (Sigma-Aldrich, #H9250; unfractionated whole histone), E. coli-derived recombinant human histone H1 (NEB, Ipswich, MA, USA #M2501), H2A (NEB, #M2502), H2B (NEB, #M2505), H3.1 (NEB, #M2503), or H4 (NEB, #M2504) (NEB's products are proteases-free, exonucleases-free, and endonucleases-free) as extracellular histones. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 with medium changes every two or three days unless otherwise specified. The cells up to the seven passages were used.

Alizarin Red S stain

The cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, followed by staining with a 1% alizarin red S solution (200 μL/well; Sigma-Aldrich, #01-2180-2) at pH6.4, adjusted with 28% ammonia solution; Nacalai Tesque, Kyoto, Japan, #02512-95) for 30 min. The cells were washed with PBS or tap water twice at each step. Stained images were captured by BZ-X810 (Keyence, Osaka, JPN). Alizarin red S was then dissolved in 10% formic acid (250 μL/well; Wako, #066-05905), and the resultant solutions were agitated for at least 30 min, and the 100 μL of these solutions was transferred into a 96-well plate and the absorbance was measured at 450 nm using a microplate reader (Infinite M nano; TECAN, Männedorf, Switzerland).

RNA extraction and RT–qPCR analyses

Total RNA was extracted using RNAiso Plus (Takara, Shiga, JPN, #9109) and reconstituted in RNase-free water. cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, JPN, #FSQ-301). RT-qPCR analysis was performed using the StepOne Plus Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) with THUNDERBIRD Next SYBR qPCR Mix (TOYOBO, #QPX-201). All processes were performed according to the manufacturer's instructions. Expression levels for all transcripts were normalized to those of *Rpl32*. The primer sequences used for this study were as follows:

mouse *Spp1* (forward) “5′- CCATGAGATTGGCAGTGATT -3′”,
(reverse) “5′- CTCCTCTGAGCTGCCAGAAT -3′”;
mouse *Tagln* (forward) “5′- CCAGACACCGAAGCTACTCT -3′”,
(reverse) “5′- ACCCTTGTTGGCCATGTTGA -3′”;
and mouse *Rpl32* (forward) “5′- AGTTCATCAGGCACCAGTCA -3′”,
(reverse) “5′- TGTCAATGCCTCTGGGTTT -3′”.

MTT assay

The viability (proliferation) was measured by an MTT assay. The cells were seeded at 10,000 cells/well in a 96-well plate (Corning, #353072). Next day, the cells were then exposed to a fresh medium with or without extracellular histones for an additional 48 h, followed by treatment with 5 mg/mL MTT (10 μL/well; Sigma-Aldrich, #M5655; dissolved by PBS) for 3 h before culture termination. After removing media, MTT was dissolved in 2-propanol (Wako, #166-04831) with 40 mM HCl (Wako, #080-01066), then shaken and protected from light for 10 min and absorbance was measured at 560 nm (Infinite M Nano, TECAN).

LDH assay

The Cytotoxicity LDH Assay Kit-WST (Dojin, Kumamoto, JPN, #CK12) was used to determine cell viability, according to the manufacturer’s protocols (non-homogeneous assay). The cells were

seeded at 20,000 cells/well in a 48-well plate. After 24 h incubation, the cells were then exposed to a fresh medium with or without extracellular histones for an additional 48 h. The supernatant (50 µL/well) was mixed with the same amount of the substrate solutions and incubated for 30 min at room temperature.

The reaction was stopped by adding half the volume of the stop solution, and the absorbance was measured at 490 nm (Infinite M Nan, TECAN). As a positive control, MOVAS cells were treated with 20 µL/well of 10% triton-X100 (Nacalai Tesque, #12967-45) for 30 min before culture termination.

Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Statistical differences were evaluated by either one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons (multiple groups) or student's t-test (two groups), using R software (Version 4.2.0). The sample size was not calculated in this study. A p-value < 0.05 was considered statistically significant.

Results

Extracellular histones accelerate Ca/Pi-dependent calcification in mouse vascular smooth muscle cells.

To investigate the effects of extracellular histones on vascular calcification, we first evaluated whether extracellular histones (unfractionated calf thymus origin) affect calcification in MOVAS cells under high Ca/Pi conditions. As expected, extracellular histones promoted Ca/Pi-dependent calcification in a dose-dependent manner (Fig.1A-B; 129% and 253% increases at 10 µg/mL and 100µg/mL, respectively, vs. Ca/Pi alone). Consistent with this, the elevated levels of *Spp1* (an osteoblast marker gene) were observed (Fig. 1C, 631% increase), in parallel with decreased levels of *Tagln* (a VSMC marker gene) (Fig. 1D, 44% decrease), when treated with 100 µg/mL histones. We also confirmed that extracellular histones enhanced Ca/Pi-dependent calcification in mouse primary VSMCs (Supplementary Fig. S1A-B).

Given that there are five families of histones (e.g., H1A, H2A, H2B, H3.1, H4) [6], we examined the effect of human recombinant histone H1, H2A, H2B, H3.1, and H4 on Ca/Pi-dependent calcification in MOVAS cells. The results showed that different histones had different effects on Ca/Pi-dependent calcification (H1A, 113%; H2A, 94%; H2B, 147%; H3.1, 466%; H4, 216% vs. Ca/Pi alone), with H3.1 being the most potent promoter of calcification in MOVAS cells under high Ca/Pi conditions (Fig. 2A-B). Although the molecular mechanism(s) underlying these findings is unknown, our data indicate that extracellular histones may be involved in Ca/Pi-induced calcification processes in mouse vascular smooth muscle cells.

As described, immune cells including macrophages were abundantly present in the aorta of CKD patients [5]. Given this, we reanalyzed published RNA-seq data from the aorta of mice fed a high phosphorus diet with unilateral nephrectomy (a model of end-stage CKD) [12]. The results showed

a tendency to increase immune cell marker genes, such as neutrophil (Itbg2 [CD18], Itgam [CD11b], Cxcr4, Mpo), macrophage (Ptprc [CD45], Fcgr1 [CD64], Adgre1[F4/80], Mertk), eosinophil (Ccr3, Il5ra, Siglecf), and basophil (Itga2b[CD41], Itga2[CD49a], Fcer1a[FcεRI]) (Supplementary Fig. S2) [12].

Extracellular histones did not affect cell death in MOVAS cells.

We attempted to demonstrate how extracellular histones promote Ca/Pi-dependent calcification in MOVAS cells. Extracellular histones are known to cause cell death in certain types, such as endothelial cells, neurons, and hair follicle progenitor cells [7–10], and factors such as apoptotic bodies derived from dead cells promote vascular calcification [11]. We, therefore, performed an LDH assay (cytotoxicity assay) to assess whether extracellular histones induce cell death in MOVAS cells. We found that extracellular histones did not affect LDH leakage, i.e., cell death, in MOVAS cells, even at concentrations that promoted Ca/Pi-dependent calcification (Fig. 3A). Because vascular calcification is known to be affected by cell density, we also examined the effect of extracellular histones on cell viability (MTT assay) in MOVAS cells. However, we could not observe any effect of extracellular histones on cell viability under normal conditions (Fig. 3B). These results suggest that the effect of extracellular histones on Ca/Pi-induced calcification in MOVAS cells is independent of their cell death.

Discussion

In this study, we found that extracellular histones promoted calcification in MOVAS cells (and primary mouse VSMCs) under high Ca/Pi conditions, possibly without cell death. As described, immune cells including macrophages were abundantly present in the aorta of CKD patients [5]. Given this, we reanalyzed published RNA-seq data from the aorta of an end-stage CKD mouse model (unilateral nephrectomy and high phosphorus diet model) [12]. The results showed a tendency to increase the genes of immune cell marker, such as neutrophil (Itbg2 [CD18], Itgam [CD11b], Cxcr4, Mpo), macrophage (Ptprc [CD45], Fcgr1 [CD64], Adgre1[F4/80], Mertk), eosinophil (Ccr3, Il5ra, Siglecf), and basophil (Itga2b[CD41], Itga2[CD49a], Fcer1a[FcεRI]) (Supplementary Fig. S2A-D) [12]. These results suggest that the release of DAMPs through extracellular trap cell death (ETosis) of neutrophils (NETosis), macrophages, eosinophils, and basophils and/or necrosis of the endothelial cells, VSMCs, and fibroblasts in aortic foci may be involved in vascular calcification. We also found that dsDNAs associated with extracellular histones were partially involved in this calcification. Taken together with the results of the re-analysis of public RNA-seq data on the CKD mouse aorta (Supplementary Fig. S2A-D), these results suggest that the release of histones through ETosis either of neutrophils, macrophages, eosinophils, or basophils and/or necrosis either of endothelial cells, VSMCs, or fibroblasts in aortic foci may be involved in vascular calcification. Since the neutrophil/lymphocyte ratio has been reported to be increased in the blood of CKD patients [13], NETosis caused by neutrophils migrating to aortic foci may be particularly implicated in vascular calcification through the release of DAMPs. Supporting this, we have also confirmed an increase in

dsDNA (probably containing histones), one of the DAMPs, in the serum of CKD model mice induced by adenine and high-phosphate diets, in which highly formed vascular calcification (Supplementary Fig. S3A-B). Thus, an increase in DAMPs, including extracellular histones and dsDNA, in aortic foci may play an important role in vascular calcification.

In this study, however, how extracellular histones promote Ca/Pi-dependent calcification in mouse VSMCs is not shown. Knockdown or knockout of the receptor of DAMPs such as Tlr4, Tlr9, and Aim2 suppressed vascular calcification [14–16], suggesting that the receptor of DAMPs involved in extracellular histones may be involved in the promotion of vascular calcification. In particular, Tlr2, Tlr4, and Clec2d have already been reported as receptors for histones [3,4], and these receptors are involved in the induction of vascular calcification by extracellular histones. However, extracted histones often contain dsDNA. Therefore, dsDNA receptors such as Aim2 and cGAS may also be involved in their promotion of vascular calcification [3]. We have also examined the effects of DNase I-treated histones (degradation of dsDNA) and have confirmed that DNase I-treated histones ameliorate the promotion of vascular calcification by extracellular histones (Supplementary Fig. S4). However, the inhibitory effect of DNase I treatment was weak, suggesting that both histones and dsDNA may be involved in the promotion of vascular calcification. It is also possible that modifications to histones and other factors that bind to histones (proteins and RNAs such as ncRNAs) may also be involved, and these factors may interact in a complex manner. According to the public single-cell analysis data of adult aorta derived from mice [17], most DAMPs receptors including histones receptors (Tlr4, Tlr7, Clec2d) are maintained at low levels under normal conditions in VSMC cells. This suggests that extracellular histones do not directly induce transformation into osteoblasts but may enhance the process of vascular calcification. Furthermore, it has been reported that TLR4, one of the DAMPs receptors, shows increased expression upon induction of vascular calcification in cultured VSMCs [14]. This suggests that VSMCs, such as MOVAS cells, are vulnerable to DAMPs signaling, particularly when DAMPs receptor expression levels are upregulated, as seen in the induction of vascular calcification. In other words, low levels of TLR4 and other DAMPs receptors may contribute to the lack of vascular calcification in the absence of histone addition, indicating that the process of vascular calcification makes VSMCs more susceptible to DAMPs signals (Fig. 1A-B). Therefore, a detailed functional analysis of these receptors will further elucidate the mechanism of vascular calcification induced by DAMPs, such as extracellular histones.

Ca/Pi-dependent vascular calcification in mouse-derived VSMCs is known to be similar to that in human VSMCs (*in vitro*) and other rodent models (*ex vivo*) [18], suggesting that our findings may be linked to vascular calcification in CKD, other vascular calcification-related various diseases and aging. Thus, we expect that extracellular histones and their associated molecules may be a therapeutic target for the treatment of vascular calcification.

Author Contributions

T.H. designed the experiments. T.H. performed all experiments and analyzed the data. T.H.

prepared and assembled the figures. T.H. and S.K. managed the project and wrote the manuscript. S.K. and D.K. provided critical reading and scientific discussions. All authors reviewed and approved the final version of this manuscript.

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Declaration of interest

The authors declare no competing financial interests.

Supplementary information

Supplementary information related to this article can be found in the online version.

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Figure Legends

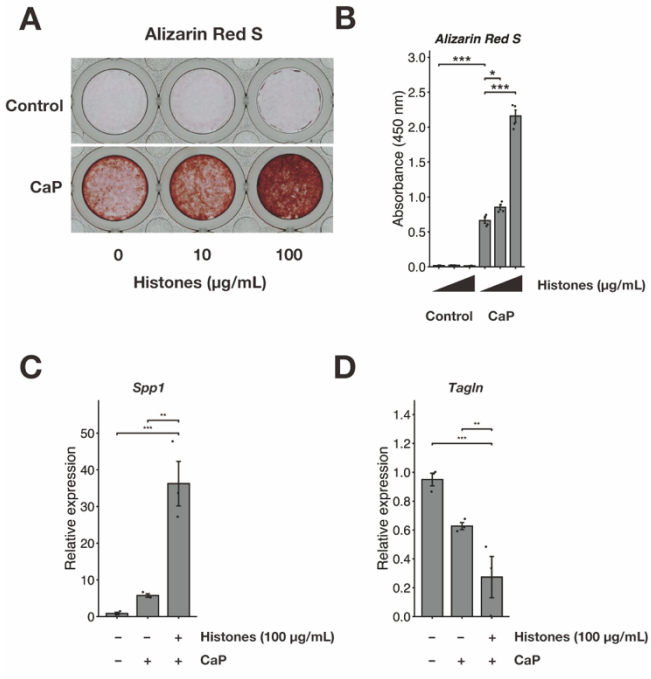

Fig. 1. Extracellular histones accelerate Ca/Pi-dependent calcification in MOVAS cells.

(**A**) The representative image of Alizarin red S stained MOVAS cells treated with calcification medium in the presence or absence of extracellular histones. n = 3 per group. (**B**) Plotted to mean the dye of Alizarin red S. n = 5 per group. (**C, D**) RT–qPCR analysis of the expression of *Spp1* (**C**) and *Tagln* (**D**). The expression levels were normalized to those of *Rpl32*. n = 3 per group. All data are presented as the mean ± SEM. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.001$. One-way ANOVA followed by Tukey's post hoc test. CaP: calcium and phosphate (Ca/Pi).

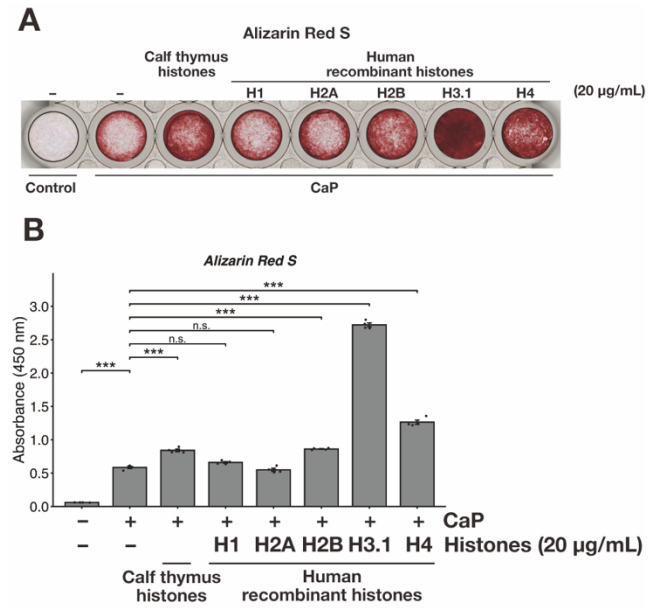

Fig. 2. Recombinant human extracellular histones accelerate Ca/Pi-dependent calcification in

MOVAS cells.

(**A**) The representative image of Alizarin red S stained MOVAS cells treated with calcification medium with extracellular histones (0 μg/mL, 10 μg/mL, 100 μg/mL). n = 3 per group. (**B**) Plotted to mean the dye of Alizarin red S. n = 5 per group. All data are presented as the mean ± SEM. ***: $p < 0.001$. n.s., not significant. One-way ANOVA followed by Tukey's post hoc test. CaP: calcium and phosphate (Ca/Pi).

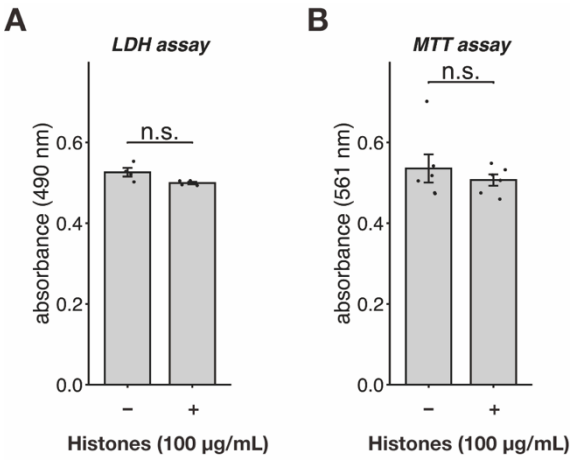

Fig. 3. Extracellular histones do not affect cytotoxicity and cell proliferation in MOVAS cells.

(**A**) LDH assay (cytotoxicity) of MOVAS cells treated with extracellular histones. n = 5 per group. (**B**) MTT assay (cell proliferation) of MOVAS cells treated with extracellular histones. n = 5 per group. All data are presented as the mean ± SEM. n.s., not significant. Student's t-test.