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Development, establishment and validation of *in vitro* and *ex vivo* assays of vascular calcification

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ABSTRACT

Objective: Vascular calcification (VC) is one major complication in patients with chronic kidney disease, with a misbalance in calcium and phosphate metabolism playing crucial role. The mechanisms underlying VC have not been entirely revealed to date. As studies aiming at the identification and characterization of the involved mediators are highly relevant, we developed a standardized operating protocol for *in vitro* and *ex vivo* approaches in this study to aiming at the comparability of these studies.

Approach and results: We analyzed *in vitro* and *ex vivo* experimental conditions to study VC. Therefore, vascular smooth muscle cells were used for *in vitro* experiments and rat aorta for *ex vivo* experiments. The degree of calcification was estimated by quantification of calcium concentrations and by von Kossa staining. As a result, a step-by-step protocol for performing experiments on VC was established. We were able to demonstrate that the degree and the location of VC in vascular smooth muscle cells and aortic rings was highly dependent on the phosphate and $CaCl_2$ concentration in the medium as well as the incubation time. Furthermore, the VC was reduced upon increasing fetal calf serum concentration in the medium.

Conclusion: In the current study, we developed and validated a standardized operating protocol for systematic *in vitro* and *ex vivo* analyses of medial calcification, which is essential for the comparability of the results of future studies.

1. Introduction

Vascular calcification is defined as deposition of calcium salts in the form of hydroxyapatite in the vascular wall. The active process of vascular calcification is a highly regulated pathophysiological event, which is physiologically relevant in bone formation as well [1]. In pathophysiological context, it is an integral characteristic of chronic kidney disease-mineral and bone disorder (CKD-MBD), a major complication associated with chronic kidney disease (CKD) [2].

Vascular calcification is a progressive disorder resulting in increased vascular stiffness, as part of cardiovascular disease contributing significantly to events [3]. In patients with CKD, the time-course of vascular calcification is accelerated beyond physiological ageing, resulting in a high cardiovascular morbidity and mortality [4,5]. Vascular calcification presents as intimal and medial calcification. While intimal calcification appears in close vicinity to lipid or cholesterol deposits, as in atherosclerotic plaque [6], medial calcification, also known as Moenckeberg's arteriosclerosis or -sclerosis, occurs independently of lipids in the medial layer of the vessel wall. Medial calcifications are characterized by mineral deposition along elastic fibers in muscle-type resistance arteries and elastic-type conduit arteries [7].

Vascular calcifications are associated with vascular smooth muscle cell (VSMC) trans-differentiation to osteoblast/chondrocyte-like cells within the vascular wall [8]. The major causes of VSMC trans-differentiation and vascular calcification involve ageing, inflammation, mechanical and oxidative stress and uremia [9], which are common in patients with CKD. Furthermore, disturbances of calcium and/or phosphate metabolism, the loss of

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Abbreviations

$CaCl_2$	Calcium chloride
CKD	Chronic kidney disease
CM	Calcifying media
FCS	Fetal calf serum
HAoSMCs	Human aortic smooth muscle cells
Na_2HPO_4	Disodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
P/S	Penicillin-streptomycin
RM	Reference medium
VC	Vascular calcification
VSMC	Vascular smooth muscle cell

natural inhibitors of vascular calcification processes or both play a crucial role in CKD with vascular calcifications [10,11]. However, the underlying molecular mechanisms are not yet completely understood [12]. There are currently many different approaches used to explore of vascular calcification processes. Therefore, standardized protocols are urgently needed.

There are currently many different protocols being used in the study of vascular calcification processes, which highly differ in the composition of calcification medium used as well as in calcification time frames being examined. A systematic approach to examine the influence of these factors on calcification has never been undertaken, although it is expected that each of these variables highly impacts on calcification extent. This complicates the comparison of study outcomes, hampers the reproducibility of data as well as reduces efficiency in calcification research through the lack of a standardized protocol. In this study, we investigated potential conditions that affect vascular calcification processes in both *in vitro* and *ex vivo* experiments on human aortic smooth muscle cells and on rat aorta. The influence of differential phosphate and calcium concentrations, incubation time as well as media supplements on vascular calcification are studied in detail. Based on these data, an optimized protocol for studying vascular calcification *in vitro* and *ex vivo* was developed and validated.

The final protocol presented will help to standardize *in vitro* and *ex vivo* approaches to investigate the processes of vascular calcification, thereby making the results of different studies comparable.

2. Materials and methods

2.1. Cell culture

For *in vitro* experiments human aortic smooth muscle cells (HAoSMCs) were used **(see Supplementary Methods)**.

2.2. Aortic ring preparation

For *ex vivo* experiments, aortas of Wistar rats were used **(see Supplementary Methods)**.

2.3. Basic incubation medium for in vitro and ex vivo experiments

The HAoSMCs and rings, respectively, were incubated using high-glucose Dulbecco's Modified Eagle's Medium (DMEM) containing already 0.9 mM phosphate, 1.8 mM calcium and 1% penicillin-streptomycin **(see Supplementary Methods)**.

2.4. Fetal calf serum (FCS) for in vitro and ex vivo experiments

Fetal calf serum (FCS) was heat inactivated and filtered. Aliquots were either stored directly at −20 °C or stored at −20 °C after an initial storage at 4 °C for 2 or 4 weeks **(see Supplementary Methods)**.

2.5. Calcification inducers for in vitro and ex vivo experiments

To investigate the effect of phosphate and calcium concentrations on vascular calcification, different concentrations of Na_2HPO_4 and NaH_2PO_4 or $CaCl_2$ were added to the DMEM with 1% P/S and 2.5 or 10% FCS just before use **(see Supplementary Methods)**.

2.6. In vitro studies using HAoSMCs and ex vivo studies using rat aortic rings

The composition of the media used in *vitro* and *ex vivo* calcification experiments is summarized in Table 1 (see Supplementary Methods).

2.7. Calcium quantification and protein measurement

The calcium content in cells and aortic rings was quantified using o-cresolphthalein complexone method. The protein content of the cells was quantified using the "Micro BCA Protein Assay Kit". The ratio of calcium content to protein content (*in vitro*) or to the dry weight of the aortic ring (*ex vivo*) was normalized to the RM **(see Supplementary Methods)**.

2.8. Histological staining

The aortic rings were stained by von Kossa staining to determine the degree of calcification in the rings. ImageJ software was used to quantify the calcified area in the adventitia and medial section, and the percentage of calcified area was calculated to the total area of the aortic ring **(see Supplementary Methods)**.

3. Statistics

see Supplementary Methods.

4. Results

To investigate the effects of phosphate and $CaCl_2$ concentrations on vascular calcification *in vitro*, HAoSMCs were incubated in the presence of media containing increased phosphate concentrations (0.9–3.8 mM) or increased $CaCl_2$ concentrations (1.8–5.0 mM), the latter at a fixed phosphate concentration of 0.9 mM or 2.8 mM (Table 1) for 7 days. Increasing the concentration of phosphate (at 1.8 mM Ca) or of $CaCl_2$ (at 0.9 mM phosphate) resulted in a gradual increase in the extent of HAoSMC calcification (Fig. 1A and B). Instead, at a phosphate concentration of 2.8 mM, increased $CaCl_2$ concentration did not have concentration-dependent effects on the calcification grade of HAoSMCs (Fig. 1C).

Next, the effects of increasing phosphate and $CaCl_2$ concentrations on vascular calcification were analyzed *ex vivo* in intact aortic preparations. Similarly, as for HAoSMCs, aortic rings were exposed to culture media containing either increasing phosphate concentrations (0.9–3.8 mM) or increasing $CaCl_2$ concentrations (1.8–5.0 mM) at a constant phosphate concentration of 0.9 mM or 2.8 mM (Table 1) for 7 days. Concentration-dependent effects were observed for both phosphate as well as for $CaCl_2$ at 0.9 mM phosphate (Fig. 1D and E). However, increasing concentrations of $CaCl_2$ at a phosphate concentration of 2.8 mM did not further enhance

Table 1
Components of media for *in vitro* and *ex vivo* experiments.

Experiment	DMEM	P/S [%]	Na_2HPO_4:NaH_2PO_4 (1:1) [mM] Final conc.	$CaCl_2$ [mM] Final conc.	FCS (fresh) [%]	FCS (2-weeks-old) [%]	FCS (4-weeks-old) [%]	Fetuin-A [mg/ml]
P and Ca conc. dependency								
RM 1	+	1	0.9	1.8	2.5	–	–	–
CM (Phosphate)	+	1	1.8, 2.8, 3.3, 3.8	1.8	2.5	–	–	–
CM ($CaCl_2$)	+	1	0.9	2.4, 3.0, 4.0, 5.0				
RM 2	+	1	2.8	1.8	2.5	–	–	–
CM (Phosphate & $CaCl_2$)	+	1	2.8	2.4, 3.0, 4.0, 5.0				
Time dependency								
RM	+	1	0.9	1.8	2.5	–	–	–
CM	+	1	2.8	1.8	2.5	–	–	–
FCS conc.dependency								
RM 2.5% FCS	+	1	0.9	1.8	2.5	–	–	–
CM 2.5% FCS	+	1	2.8	1.8	2.5	–	–	–
RM 10% FCS	+	1	0.9	1.8	10	–	–	–
CM 10% FCS	+	1	2.8	1.8	10	–	–	–
FCs age dependency								
RM fresh FCS	+	1	0.9	1.8	2.5	–	–	–
CM fresh FCS	+	1	2.8	1.8	2.5	–	–	–
RM 2-weeks-old FCS	+	1	0.9	1.8	–	2.5	–	–
CM 2-weeks-old FCS	+	1	2.8	1.8	–	2.5	–	–
RM 4-weeks-old FCS	+	1	0.9	1.8	–	–	2.5	–
CM 4-weeks-old FCS	+	1	2.8	1.8	–	–	2.5	–
VC inhibitor experiments								
RM	+	1	0.9	1.8	2.5	–	–	–
CM	+	1	2.8, 3.8	1.8	2.5	–	–	–
CM + fetuin-A	+	1	2.8, 3.8	1.8	2.5	–	–	1

calcium content (Fig. 1F).

For visualization of the calcified area in the aortic rings von Kossa staining was used (Fig. 2A–C), and the calcified areas within media and adventitia were quantified as percentage of the total aortic ring area (Fig. 2D–F). Incubation of aortic rings in medium with increasing phosphate concentrations caused an increasing degree of calcification specifically in the media but not in the adventitia of aortic rings (Fig. 2D, white (media) vs. black (adventitia) bars). In contrast, increased $CaCl_2$ concentrations in the incubation medium decreased the degree of medial calcification at low (0.9 mM) and especially high (2.8 mM) phosphate levels, whereby the higher the $CaCl_2$ levels, the less medial calcification was observed (Fig. 2E and F, white bars). Instead, calcification in the adventitia increased upon increasing $CaCl_2$ concentrations in a dose-dependently way (Fig. 2E and F, black bars).

Next, the effect of incubation time on the degree of vascular calcification was analyzed *in vitro* as well as *ex vivo*. As we are particularly interested in medial calcification being associated with cardiovascular risk [13], a calcification medium containing 2.8 mM phosphate and 1.8 mM $CaCl_2$ (CM) was used to induce medial calcification of the aortic rings (Table 1). HAoSMCs were incubated for 3, 5, 7, 9 and 14 days, respectively, to analyze the effect of the incubation period on the degree of the calcification *in vitro*. A time-dependent increase in calcium content in HAoSMCs is shown in Fig. 3A.

A comparable effect was observed *ex vivo* when aortic rings were incubated in the presence of CM for 3, 5, 7, and 14 days, respectively (Fig. 3B). The time-dependent induction of medial calcification in aortic rings was confirmed by staining the calcified area with von Kossa staining and calculating the calcified area within the media as percentage of the total aortic ring area (Fig. 3C and D).

Next, we analyzed whether different regions of the aorta are calcifying equally. Therefore, the thoracic and abdominal aorta of rats were gently dissected and cut into 30 rings. The aortic rings were incubated for 7 days in the presence of RM or CM (Table 1). Fig. 3E shows the corresponding results of a representative aorta. A constant degree of calcification in the thoracic aorta (aortic rings 1–15) was detected. In contrast, a modified degree of calcification in the abdominal aorta region (aortic rings no. 16–30) was quantified, whereby a particularly increased calcification was noticed at the branching points (aortic rings no. 16, 20, 22, 29).

Next, the effect of the media component fetal calf serum (FCS) on the degree of vascular calcification was analyzed. HAoSMCs were incubated for 7 days in the presence of CM containing 2.5% or 10% FCS or the corresponding RM (Table 1). Both CMs induced calcification in HAoSMCs. However, incubation of cells in CM containing 2.5% FCS showed a higher degree of calcification compared to the CM containing 10% FCS (Fig. 4A). A comparable effect was observed when analyzing calcification extent in aortic rings through calcium measurement (Fig. 4B) as well as von Kossa staining (Fig. 4C). After von staining the calcified regions and calculating the calcified area in the media as percentage of the total aortic ring area, less calcification was observed in aortic rings incubated in the presence of 10% FCS medium compared to 2.5% FCS medium (Fig. 4D).

To analyze the effect of the aging of FCS on vascular calcification processes *in vitro*, HAoSMCs were incubated for 7 days in the presence of CM containing 2.5% fresh, 2- or 4-weeks-old FCS or the corresponding RM (Table 1) (Fig. 4E). A comparable calcification extent was observed in the presence of FCS up to an age of 4 weeks compared to fresh FCS. This effect was validated *ex vivo* in aortic rings, as displayed by quantifying calcification extent through calcium measurement (Fig. 4F) as well as through von Kossa staining (Fig. 4G).

Next, we analyzed how the degree of vascular calcification influences the effect of a vascular calcification inhibitor on the extent of vascular calcification. *In vitro*, HAoSMCs or *ex vivo*, aortic rings were incubated for 7 days with CM containing 2.8 mM or 3.8 mM phosphate in the absence or presence of 1 mg/ml fetuin-A. *In vitro* the calcium content was reduced by 30% in cells incubated in the CM containing 2.8 mM phosphate and 1 mg/ml fetuin-A compared to cells incubated in the presence of 2.8 mM phosphate. In contrast, no significant inhibition of calcification by fetuin-A was observed in

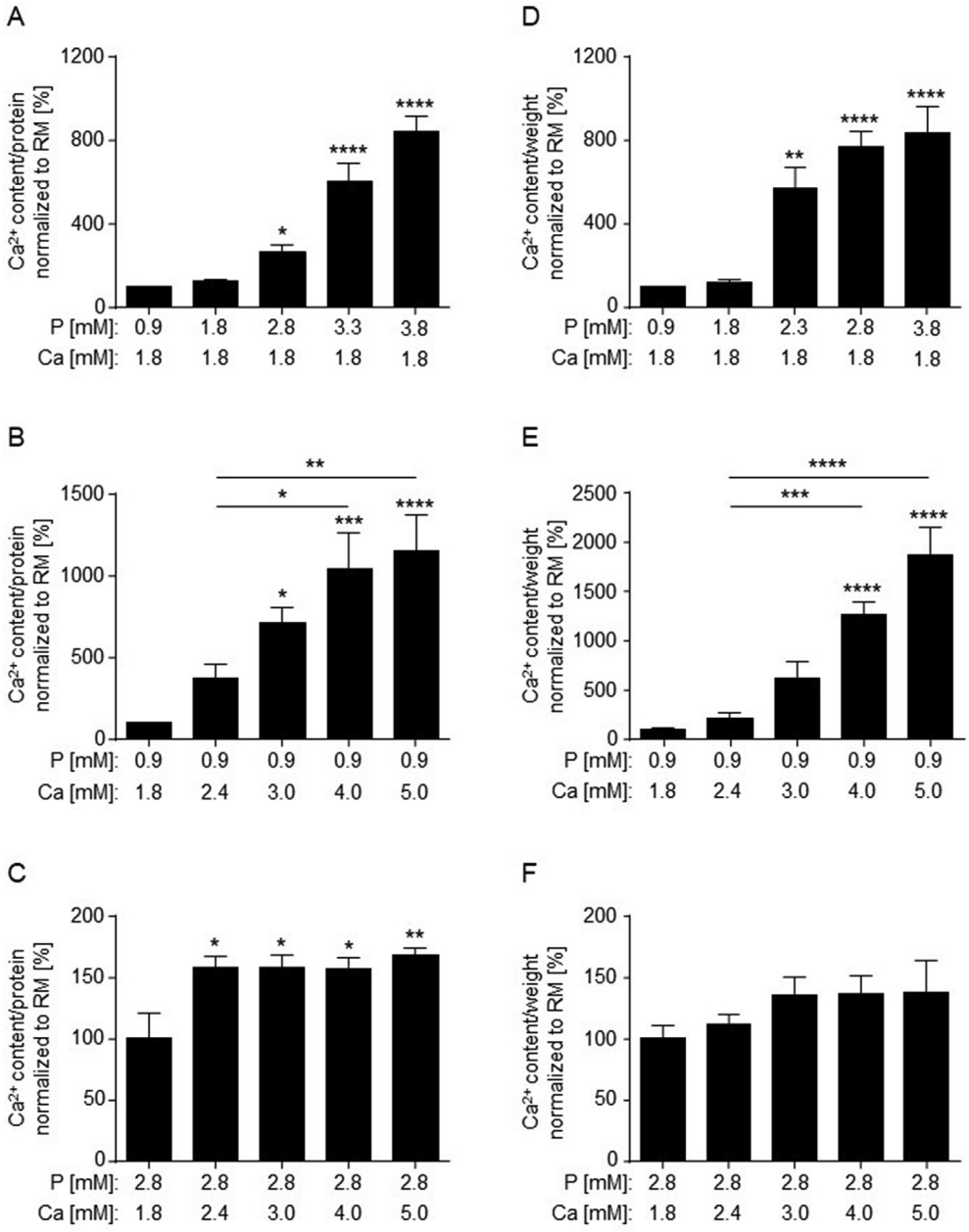

Fig. 1. The extent of vascular calcification is dependent on phosphate and $CaCl_2$ concentrations. HAoSMC and aortic rings were incubated for 7 days in medium in the presence of increasing phosphate concentrations (A, D) or increasing $CaCl_2$ concentrations in the presence of low (B, E) vs. high phosphate concentration (C, F). The ratio of calcium content to protein content, or calcium content to the dry weight of the aortic ring, was normalized to that of the corresponding RM. Data shown as means ± SEM (n = 3–9 independent experiments for each treatment). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 compared with RM based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test.

cells incubated in the CM containing 3.8 mM phosphate (Supplementary Fig. 1A). An inhibition of calcification in the presence of fetuin-A was detected *ex vivo* in both CM; however, the reduction in calcium content was less after incubation of the aortic rings in medium containing the increased phosphate concentrations (Supplementary Fig. 1B). This effect was confirmed when the extent of medial calcification in aortic rings was quantified after von Kossa staining (Supplementary Figs. 1C–D).

5. Discussion

A misbalance in the calcium-phosphate metabolism plays an essential role in the development and aggravation of vascular calcifications [10,11]. In healthy subjects, phosphate levels range between 0.81 and 1.45 mM, and calcium levels are in the range of 2.1–2.6 mM[14]. Both phosphate and calcium plasma levels are elevated in end-stage renal disease up to 2.85 mM and 2.95 mM, respectively [15]. End-stage renal disease patients are characterized by extensive and accelerated vascular calcification compared to the general population [16]. Ca ions and phosphate are widely used *in vitro* and *ex vivo* to induce calcifications in experimental conditions. For example, a large number of studies have demonstrated that incubation of VSMCs in the presence of increased phosphate concentrations increases vascular calcification [16,17], although a systematic approach to study the influence of each factor was, to our knowledge, never undertaken. In the present study, we were

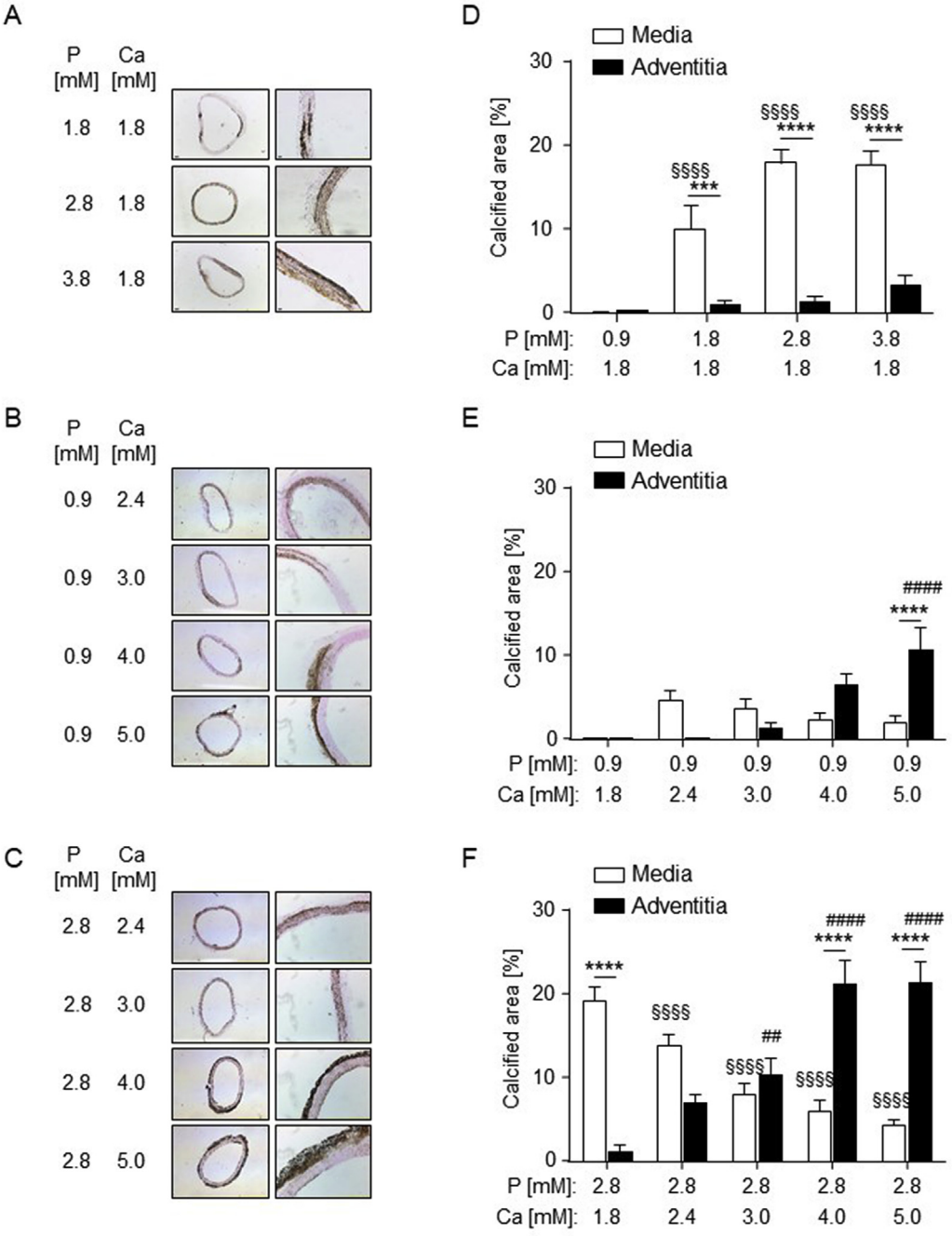

Fig. 2. Increased phosphate concentrations in the medium induce medial calcification in aortic rings. Aortic rings were incubated for 7 days in the presence of increasing phosphate concentrations (A, D), increasing $CaCl_2$ concentrations in the presence of low (B, E) vs. high phosphate concentration (C, F). The calcified aortic areas were visualized by von Kossa staining. Representative pictures are given (original magnification ×40; section of ring original magnification ×200) (A–C) and the calcified areas within the media (white bars) and adventitia (black bars) were quantified as percentage of the total aortic ring area (D–F). Data shown as means ± SEM (n = 3–7 independent experiments for each treatment). ***P ≤ 0.001, ****P ≤ 0.0001 represent a significant different between media and adventitia. §§P ≤ 0.01, §§§P ≤ 0.001 and §§§§P ≤ 0.0001 represents a significant difference between media compared to RM. ##P ≤ 0.01, ###P ≤ 0.001 and ####P ≤ 0.0001 represents a significant difference between adventitia compared to RM. All significants are based on two-way ANOVA. Bonferroni's multiple comparisons were used as a post-test.

able to demonstrate a concentration-dependent induction of vascular calcification by increasing the phosphate or $CaCl_2$ concentration in the medium. Calcification was induced when reaching a phosphate concentration of 2.8 mM or above, or a $CaCl_2$ concentration of 3 mM or above. These concentrations correspond to the phosphate and calcium levels observed in the serum of CKD patients. Furthermore, we were able to demonstrate that if a high phosphate concentration of 2.8 mM is present in the medium, increasing $CaCl_2$ concentrations in the medium does not lead to a further dose-dependent increase in the total degree of vascular calcification (Fig. 1). Even though a concentration-dependent increase in calcium content was detected by increasing phosphate or $CaCl_2$ concentrations in the medium, only an increase in phosphate concentrations lead to increased medial calcification. In contrast, increased $CaCl_2$ concentrations in the medium had an inhibitory effect on medial calcification, both in the presence of low as well as

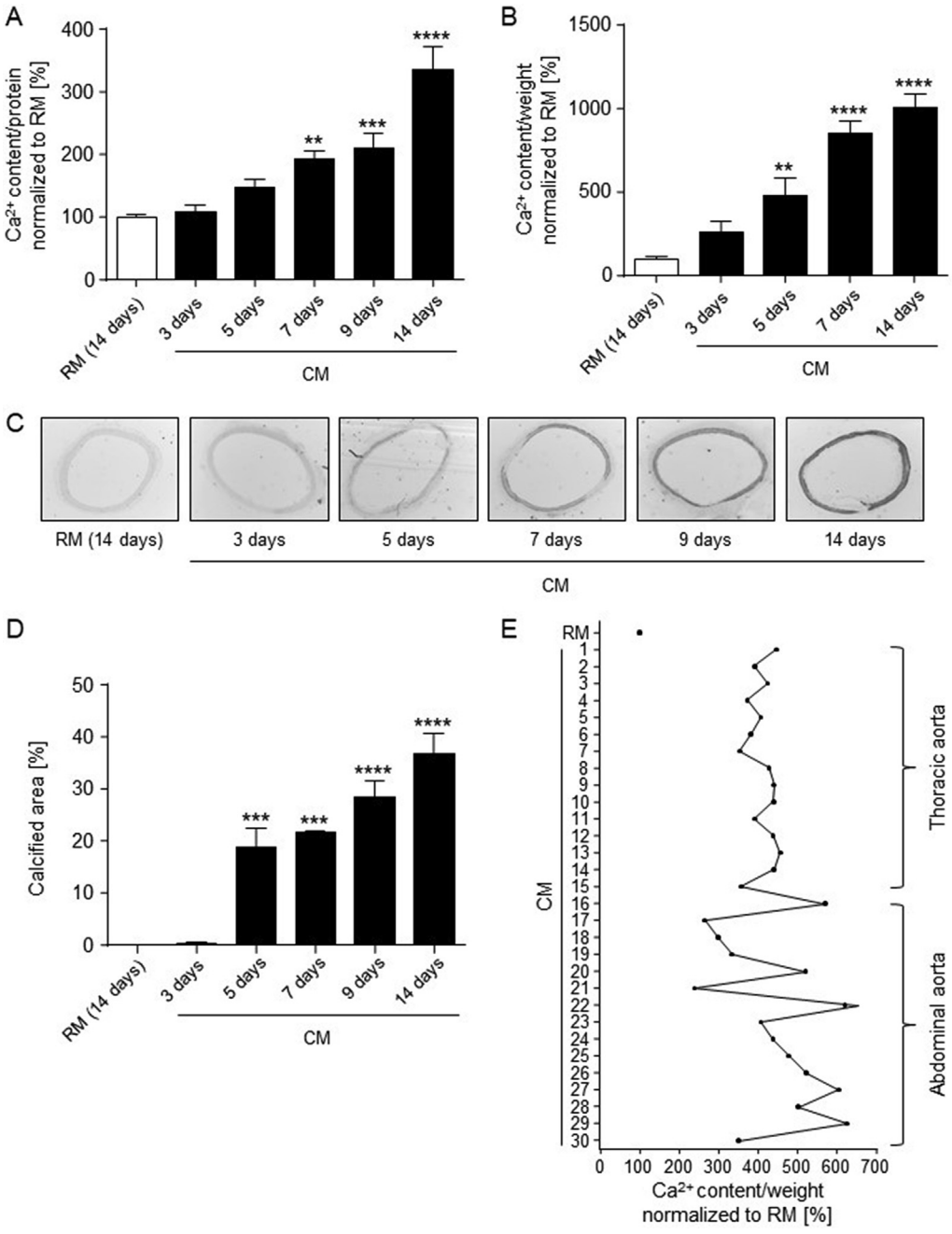

Fig. 3. The extent of medial calcification is dependent on the incubation time. HAoSMCs (A) or aortic rings (B) were incubated for increasing time periods in CM or for 14 days RM. The ratio of calcium content to protein content, or calcium content to the dry weight of the aortic ring, was normalized to that of RM. Aortic rings were stained by von Kossa staining. Representative pictures are given (original magnification ×40) (C), and the calcified area within the media was quantified as percentage of the whole aortic ring area (D). The whole rat aorta was gently dissected and cut into 30 rings. The aortic rings were incubated for 7 days in the presence of CM. One ring was incubated in the presence of RM. Calcium content was quantified and the ratio of calcium to the dry weight of the aortic ring was normalized to that of RM. A representative graph of three independent experiments is shown (E). Data shown as means ± SEM (n = 3–5 independent experiments for each treatment). **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 compared with RM based on one-way ANOVA. Bonferroni's multiple comparisons were used as a post-test.

high phosphate levels (Fig. 2, white bars). Calcification of the adventitia, on the other hand, was enhanced by increased $CaCl_2$ concentrations in the medium. This effect was even amplified in the presence of increased phosphate concentration (Fig. 2, black bars). Vascular calcification is an active process associated with VSMC transdifferentiation to osteoblast/chondrocyte-like cells within the vascular wall and promoted by inorganic phosphate [8,18]. Therefore, we hypothesize that an increased phosphate concentration in the medium may impact on the differentiation processes of VSMCs located in the medial part of the vessel, resulting in medial calcification. The concept of phosphate-induced medial calcification is also supported by previous *in vivo* experiments demonstrating that feeding uremic mice with phosphate induces medial arterial calcification [19]. How increased $CaCl_2$ concentrations reduce medial calcification and trigger adventitial calcification instead, remains currently unclear. It may be speculated that, compared to phosphate, increased $CaCl_2$ concentrations promote the formation of different mineralization products with preferential nucleation

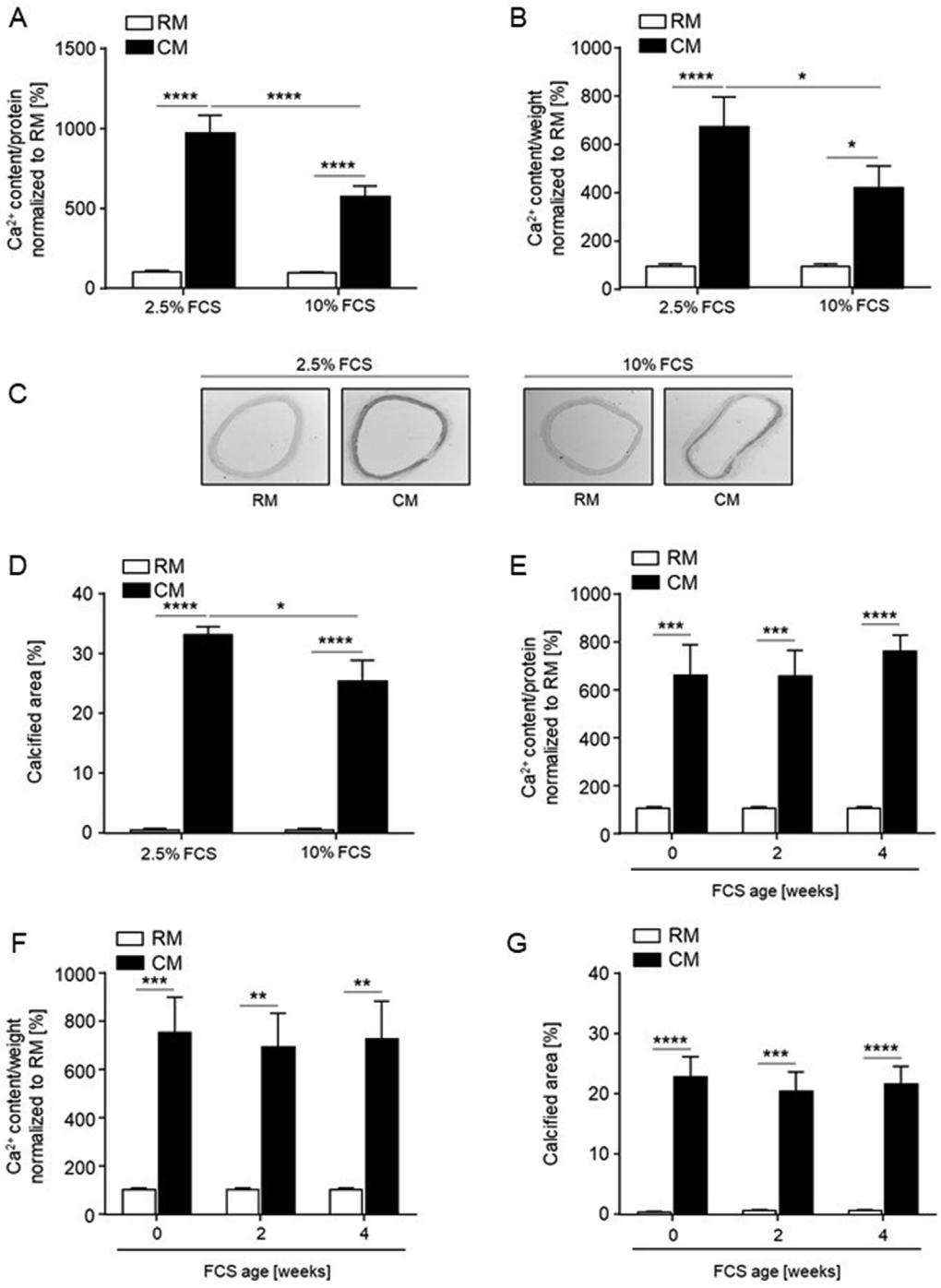

Fig. 4. Medial calcification extent is dependent on FCS concentration but is independent of the aging of FCS. HAoSMCs (A) or aortic rings (B) were incubated for 7 days in the presence of CM containing 2.5% or 10% FCS, or in the presence of corresponding RM. The ratio of calcium content to protein content, or calcium content to the dry weight of the aortic ring, was normalized to that of RM. Aortic rings were stained by von Kossa staining. Representative pictures are given (original magnification ×40) (C), and the calcified area within the media was quantified as percentage of the whole aortic ring area (D). HAoSMCs (E) or aortic rings (F) were incubated for 7 days in the presence of CM containing 2.5% fresh (0) or 2- or 4-weeks-old FCS or in corresponding RM. The ratio of calcium content to protein content, or calcium content to the dry weight of the aortic ring, was normalized to that of RM. Aortic rings were stained by von Kossa staining and the calcified area within the media was quantified as percentage of the whole aortic ring area (G). Data shown as means ± SEM (n = 3–5 independent experiments for each treatment). *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 compared with RM based on two-way ANOVA. Bonferroni's multiple comparisons were used as a post-test.

and/or deposition in the adventitial region. This may reduce phosphate accumulation and calcification nucleation in the media, thereby reducing medial calcification.

Based on these differential effects of phosphate and calcium on the vascular location of calcification induction, quantification of only calcium content in aortic rings does not sufficiently inform on calcification responses when not combined with a histological analysis of calcification localization through von Kossa staining. As demonstrated in Fig. 3 A-D, an incubation period of at least 7 days is essential to induce calcification in both *in vitro* as well as *ex vivo*

conditions using a calcifying medium containing 2.8 mM phosphate, 1.8 mM $CaCl_2$, with longer incubation times further increasing calcification extent. This time-dependency is in line with previous data using VSMCs incubated for 8, 10 and 12 days in the presence of 1.0, 2.0 or 3.0 mM phosphate, and quantifying calcium as read-out parameter [20]. In addition to P/Ca dose- and time-dependency of calcification processes, we analyzed the effect of differential aortic regions used for calcification processes, since, to the best of our knowledge, the impact of vascular localization on the degree of calcification in the entire aorta has not yet been investigated *ex vivo*. In the present *ex vivo* study, we demonstrate that the degree of calcification is not uniform within the whole aorta. While in the thoracic region a relatively constant calcification level was observed, the abdominal area showed an uneven distribution of calcification extent, with an increased calcification particularly noticeable at the branching points (Fig. 3E). In these regions, the blood flow is more turbulent, which has been associated with endothelial dysfunction [21]. Since endothelial dysfunction is associated to calcification [22], these branching points with turbulent blood flow may render these areas more prone to vascular calcification, also in the *ex vivo* situation. Furthermore, *in vitro* and *ex vivo,* vascular calcification was shown to be dependent on FCS concentration in the medium, with a reduced calcification extent in the presence of high vs. low FCS concentrations. Being a component of the liquid fraction of clotted blood from fetal calves, depleted of cells, fibrin and clotting factors, FCS contains a large number of nutritional and macromolecular factors essential for cell growth and survival [23]. Furthermore, serum contains important calcification inhibitors, as fetuin-A [24] and matrix gla protein (MGP) [25]. Fetuin-A is an abundant protein in FCS besides albumin as main component of FCS [24]. Fetuin-A is an inflammation-related Ca-regulatory glycoprotein with multiple Ca-binding sites, and is a well-known inhibitor of vascular calcification [26]. Thus, reduced calcification in the presence of increased FCS concentrations may be caused by the increased amount of calcification inhibitors as fetuin-A present. In addition to FCS concentrations, we analyzed the effect of aging of FCS on vascular calcification extent. However, in contrast to the strong effect of FCS concentration on calcification extent, aging of FCS did not impact on calcification degrees (Fig. 4E–G). Still, for our experiments, FCS was heat-inactivated and filtered, and aliquots were stored directly at −20 °C to prevent aging. It is also important to point out that FCS from different lots, even from the same company, might have a slightly higher or lower calcification inhibition potential due to biological variations in the molecular composition of FCS. Therefore, it is suggested to use FCS from the same company and from the same lot in the frame of one set of calcification experiments. Taking all these parameters into account and aiming at one protocol for *in vitro* experiments with HAoSMCs as well as *ex vivo* experiments with aortic rings, a standardized calcification protocol was synthesized to study medial calcification (Supplementary Fig. 2). In this protocol, calcification is induced by incubating HAoSMCs or rings of rat thoracic aorta for 7 days in high-glucose DMEM medium containing 1% P/S, 2.5% FCS and a final concentration of 1.8 mM $CaCl_2$ and 2.8 mM phosphate. As reference medium, final phosphate and calcium concentrations were 0.9 mM and 1.8 mM, respectively. Calcification analysis in HAoSMCs is performed by calcium measurement using the o-cresolphthalein complexone method, whereas analyses in aortic rings require a combination of calcium measurement by the o-cresolphthalein complexone method as well as von Kossa staining to enable a distinguishment between medial vs. adventitial calcification. Furthermore, exactly this additional information on calcification location using aortic rings underlines the need to confirm study results from *in vitro* studies also *in ex vivo* experiments.

In conclusion, in the current study, we developed and validated a standardized operating protocol (SOP) for systematic *in vitro* and *ex vivo* analyses of medial calcification (Supplementary material), which is essential for the comparability of the results of future studies.

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Declaration of competing interest

The authors have no conflicting interests to declare.

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J.H. and S. O-A designed the study; J.H. and S.B. conducted the experiments; S. O-A. and J.J. analyzed the data, prepared the figures and tables and wrote the paper. J.J., H.N. and M.B. contributed by drafting and critically revising the paper. All authors approved the final version of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.05.085.

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