Zinc As A Factor Affecting Serum Calcification Propensity in Patients With Type 2 Diabetes Mellitus

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Abstract

Background: Zinc inhibits vascular calcification *in vivo* and *in vitro*. Patients with type 2 diabetes mellitus show hypozincemia and are at an elevated risk of cardiovascular events. Recently, the *in vitro* test (T_{50}-test) was developed for the determination of serum calcification propensity. This cross-sectional study investigated the association between serum zinc and T_{50} in type 2 diabetes mellitus patients and the effect of zinc on T_{50} *in vitro.*

Methods: The subjects were 132 type 2 diabetes mellitus patients with various kidney function. We measured T_{50} levels by the established nephelometric method.

Results: The median (interquartile range) levels of T_{50} and serum zinc were 306 (269 to 332) min, and 80.0 (70.1 to 89.8) μg/dL, respectively. Serum zinc level was significantly and positively correlated with T_{50} (r_s = 0.219, p = 0.012). This association remained significant in multivariable-adjusted analysis, and was independent of known factors including phosphate, calcium, and magnesium. Renal function and glycemic control were not significantly associated with T_{50}. Finally, addition of physiological concentration of exogenous zinc chloride significantly increased the serum T_{50} i*n vitro*.

Conclusions: This is the first report to investigate the association between serum calcification propensity and zinc levels in type 2 diabetes mellitus patients. Our data suggest that serum zinc is an independent factor that inhibits serum calcification propensity.

Background

Vascular calcification is common and contributes to cardiovascular mortality in patients with type 2 diabetes mellitus [1, 2], and those with chronic kidney disease [3, 4] . The excess cardiovascular morbidity and mortality in those patients could be explained by redistribution and/or overload of calcium and phosphorus as well as imbalanced-calcification regulators [3, 5] in these conditions. The mechanism of vascular calcification is supposed to be due to ectopic deposition of hydroxyapatite [6, 7] induced by increased calcium-phoshprous product (Ca × P) in serum [8, 9], dedifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells [10, 11] and accumulation of degenerative extracellular matrix [12]. It is hypothesized that diabetic condition and renal dysfunction share some common causal pathways leading to vascular calcification.

In serum, precipitation of supersaturated calcium and phosphate is prevented by the formation of amorphous primary calciprotein particles (CPPs) [13, 14]. Primary CPPs spontaneously convert into secondary CPPs, containing crystalline hydroxyapatite [13, 14]. The *in vitro* test (T_{50}-test) for the determination of serum calcification propensity was developed [15]. This assay measures time required for primary CPPs to transform into secondary CPPs in the presence of supersaturating doses of calcium and phosphate, which increase turbidity of samples. T_{50} can be measured by laser light scatter in turbid samples using nephelometry. Thus, a shorter T_{50} means a higher calcification propensity. Previous

studies have shown that lower T_{50} predicts vascular stiffness progression and all-cause mortality in patients with chronic kidney disease stage 3 and 4 [16], and all- cause mortality and cardiovascular composite endpoint in hemodialysis patients.[17]. A Lower T_{50} was also shown to predict cardiovascular and all-cause mortality in renal transplant recipients [18, 19].

T_{50} is depended on the complex interplay of pro-calcifying (*i.e.* calcium and phosphate) and anti-calcifying serum components (*i.e.* magnesium and albumin) [15]. Among them, a higher serum phosphate level was the factor most closely associated with lower T_{50}[17, 20]. Phosphate has been reported to induce calcification of VSMCs i*n vitro* [21]. Hyperphosphatemia is a risk factor for vascular calcification and cardiovascular mortality, not only in patients with chronic kidney disease [22], but also in the general population [23]. Thus, suppression of phosphate-induced vascular calcification is clinically important.

Zinc is an essential micronutrient that plays catalytic, structural, and regulatory roles [24]. Recently, zinc was found to inhibit phosphate-induced vascular calcification, *in vitro* and in vivo [25]. In human, zinc level in blood was reported to be lower in patients with type 2 diabetes mellitus compared to non-diabetic subjects [26-28]. So far, however, the role of zinc in serum calcification propensity is not established.

These previous studies raise the hypothesis that zinc could be one of the factors affecting with serum calcification propensity. To test the hypothesis, we examined the association between serum zinc and T_{50} levels and the effect of increasing zinc concentration on T_{50}*in vitro.*

Methods

Ethics statement

This study followed the ethical guidelines for medical and health research involving human subjects by the Japanese Ministry of Health, Labour and Welfare, and the Declaration of Helsinki. This study was approved by the Ethics Committee of Osaka City University Graduate School of Medicine (approval No. 4100). Opt-out option for informed consent was performed as explained in instructions posted on the website of the institution.

Study design and participants

This study comprised of two parts. The first part was a cross-sectional study using clinical data derived from our previous study including 143 patients with type 2 diabetes mellitus [29]. The inclusion and exclusion criteria for the clinical study were described as previously [29]. For this analysis, we excluded 11 participants because data of T_{50} and zinc were not available. Finally, 132 patients were included in the present study. The second part was an *in vitro* study in which the effect of increasing zinc concentration on T_{50} assay was examined.

Determination of calcification propensity

As previously reported [15], calcification propensity was evaluated by overloading of calcium and phosphate into sera ex vivo. Spontaneous transformation of primary to secondary CPPs can increase turbidity in each serum in the presence of supersaturated solution with time. The light scattering intensity accompanied by progressive turbidness was measured by time-resolved nephelometry. T_{50} was determined one-half maximal transition time, that is, a half-time of transformation from primary to secondary CPPs. According to the original method, we prepared three stock solutions as follows: (1) NaCl solution: 140mM NaCl, (2) Calcium solution: 40 mM $CaCl_2$+100 mM HEPES+140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C, and (3) Phosphate solute on: 19.44 mM Na_2HPO_4+4.56 mM NaH_2PO_4+100 mM HEPES+140 mM NaCl pH-adjusted with 10 M NaOH to 7.40 at 37°C. In the 96-well plates, 20 µl of NaCl stock solution and 80 µl of serum were mixed in each well and then shacked for 1 minute. Subsequently, 50 µl of phosphate stock solution and 50 µl of calcium stock solutions were added and shacked for 1 minute, automatically, in the pre-warmed thermo-constant room at 34.5°C. The final concentrations of calcium and phosphate in each sample were 10 mM and 6 mM, respectively. T_{50} was determined in duplicate over 600 minutes per one measurement using a nephelometer (Nephelostar Plus^R, BMG Labtech, Ortenberg, Germany).

All serum samples were measured in a blinded manner. Serum samples from healthy volunteers and dialysis patients were also measured as quality control in serum calcification assay. The coefficients of variation (CV) of inter- and intra-assay were 4.4 % and 4.5 % in healthy control serum, 3.2 % and 4.5 % in hemodialysis control serum.

Where indicated, zinc chloride ($ZnCl_2$) (0, 10, and 20 µM) was added to the serum samples, respectively.

Blood and urine sampling and Measurements

Serum zinc levels were measured by a commercial laboratory (SRL Co., Ltd., Tokyo, Japan). Renal function was assessed by estimated glomerular filtration (eGFR) using a formula for the Japanese [30]. In this study serum calcium denotes calcium level adjusted for serum albumin according to Payne et al [31]. Urinary albumin to creatinine ratio was calculated as an index of albuminuria. Other measurements were obtained using routine laboratory methods at Osaka City University Hospital.

Other clinical information

We collected information on age, sex, height, weight, duration of diabetes, current medications, past history of cardiovascular disease (coronary artery disease, peripheral artery disease, aortic disease, and congestive heart failure requiring hospitalization), smoking habit, and laboratory data by asking the participants and/or by reviewing their medical records.

The diagnosis of type 2 diabetes mellitus was based on medical record and the criteria for diabetes mellitus as defined in the Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus [32].

Statistics

In clinical study, we summarized continuous variables as medians (interquartile ranges, IQRs) and categorical variables as numbers and percentages. Correlations were analyzed according to the nonparametric Spearman's rank correlation test. Independent associations between the variables and T_{50} were assessed by multiple regression analysis. In in vitro experiments in which $ZnCl_2$ was added, T_{50} was expressed mean (SD) of the triplicate determinations, and comparison was made by one-way analysis of variance followed by Tukey's test. These statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA) or JMP software version 10 (SAS Institute, Inc., Cary, NC, USA). P-values < 0.05 by two-sided tests were considered statistically significant.

Results

Clinical characteristics of the type 2 diabetes patients

Table 1 summarizes the clinical characteristics of the 132 patients with type 2 diabetes mellitus. They all had type 2 diabetes mellitus, although the eligibility criteria did not exclude patients with type 1 diabetes. The age [median (interquartile range)] was 71 (65 to 75) years, and 59.1% of the participants were men. The known duration of diabetes mellitus was 13 (8 to 20) years. Their eGFR [59.0 (37.6 to 73.9) mL/min/1.73 m^2] and urinary albumin to creatinine ratio [17 (6 to 212) mg/gCr] showed wide distributions. T_{50} and serum zinc levels were 306 (269 to 332) min, and 80.0 (70.1 to 89.8) μg/dL, respectively.

Table 1
Clinical characteristics of the study participants (n = 132)

Measurement	Median (IQR) or Percentage
Age (years)	71 (65–75)
Male/female, N(%)	78 (59.1) / 54 (40.9)
Body mass index (kg/m^2)	24.5 (21.8–27.0)
T_{50} (min)	306 (269–332)
eGFR (mL/min/1.73 m^2)	59.0 (37.6–73.9)
Creatinine (mg/dL)	0.87 (0.69–1.36)
Blood urea nitrogen (mg/dL)	17 (15–23)
Serum albumin (g/dL)	4.1 (3.8–4.3)
Fasting plasma glucose (mg/dL)	119 (99–143)
HbA1c (%)	8.0 (7.1–9.2)
Corrected calcium (mg/dL)	9.4 (9.2–9.7)
Phosphate (mg/dL)	3.8 (3.4–4.2)
Magnesium (mg/dL)	2.1 (2.0-2.3)
Zinc (μg/dL)	80.0 (70.1–89.8)
Whole PTH (pg/mL)	21.6 (16.4–31.2)
Intact PTH (pg/mL)	36 (27–54)
Urine albumin to creatinine ratio (mg/gCr)	17 (6–212)
Systolic blood pressure (mmHg)	125 (110 – 38)
Diastolic blood pressure (mmHg)	65 (60–74)
Current smoker (%)	65 (49.2)
Use of mediations	
Antihypertensive (%)	79 (59.8)
Statin (%)	64 (48.4)
Insulin (%)	63 (47.7)

The table gives number and percentage for categorical variables and median (IQR) for continuous variables. Abbreviations are: IQR, interquartile range; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; PTH, para-thyroid hormone.

Measurement	Median (IQR) or Percentage
Anti-diabetic agent (%)	98 (74.2)
Complications	
Retinopathy (%)	37 (28.2)
Neuropathy (%)	66 (50.0)
Any prior cardiovascular disease (%)	21 (15.9)

The table gives number and percentage for categorical variables and median (IQR) for continuous variables. Abbreviations are: IQR, interquartile range; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; PTH, para-thyroid hormone.

Correlations between serum calcification propensity and clinical factors

Table 2 shows the unadjusted correlations between T_{50} levels and various clinical parameters in type 2 diabetes patients. While T_{50} was positively correlated with zinc (r_s = 0.219, p = 0.012, Fig. 1), eGFR (r_s = 0.199, p = 0.022), and fasting plasma glucose (r_s = 0.282, p = 0.001), it was not significantly correlated with serum magnesium or hemoglobin A1c. T_{50} level was negatively correlated with urinary albumin-creatinine ratio (r_s = − 0.247, p = 0.004), blood urea nitrogen (r_s = – 0.213, p = 0.011), and serum phosphate (r_s = − 0.227, p = 0.009).

Table 2
Correlation of T_{50} with clinical factors in diabetic patients

Clinical Variables	Correlation with T_{50}	
	r_s	p
Age	**−0.222**	**0.010**
Body mass index	0.013	0.886
eGFR	**0.199**	**0.022**
Creatinine	−0.170	0.051
Blood urea nitrogen	**−0.213**	**0.011**
Serum albumin	**0.313**	**0.0003**
Fasting plasma glucose	**0.282**	**0.001**
HbA1	0.166	0.057
Corrected calcium	0.132	0.132
Phosphate	**−0.227**	**0.009**
Magnesium	0.113	0.195
Zinc	**0.219**	**0.012**
Whole-PTH	−0.117	0.183
Intact-PTH	−0.110	0.183
Urine albumin to creatinine ratio	**−0.247**	**0.004**
Data include the Spearman's correlation coefficient (r_s-value) and the levels of significance (p-value) (bolded if $p < 0.05$).		
Abbreviations are: eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; PTH, para-thyroid hormone.		

Independent association between serum calcification propensity and zinc

We examined whether serum zinc level was a factor associated with serum T_{50}, independent of traditional mineral makers including phosphate, calcium and magnesium, using multiple regression analysis (Table 3). Model 1 included age, sex, any prior cardiovascular disease, current smoking, urinary albumin creatinine ratio, eGFR, corrected calcium, phosphate, magnesium, zinc, and hemoglobin A1c as explanatory variables, with hemoglobin A1c being replaced by plasma glucose in Model 2. Serum Zinc level was found to be associated significantly and positively with T_{50} in both Model 1($\beta = 0.213$, $p = 0.038$) and Model 2 ($\beta = 0.229$, $p = 0.024$).

	Model 1		Model 2	
	b	*p*	b	*p*
Age	–0.077	0.412	–0.056	0.544
Sex (female = 0. male = 1)	0.042	0.633	0.035	0.692
Any Prior cardiovascular disease	–0.143	0.112	–0.139	0.117
Current smoking (no =0, yes = 1)	–0.142	0.094	–0.135	0.105
eGFR	0.172	0.155	0.096	0.438
Urinary albumin-creatinine ratio	0.004	0.968	0.028	0.783
Corrected calcium	**0.228**	**0.010**	**0.199**	**0.021**
Phosphate	**–0.328**	**0.0007**	**–0.317**	**0.0008**
Magnesium	**0.226**	**0.009**	**0.216**	**0.011**
Zinc	**0.213**	**0.038**	**0.229**	**0.024**
HbA1c	–0.054	0.537	-	-
Fasting plasma glucose	-	-	0.171	0.053
R^2	0.244 ($p < 0.001$)		0.265 ($p < 0.001$)	

Table 3.Factors associated with serum calcification propensity (T_{50}) in 132 type 2 diabetes patients

Data are the standard regression coefficients (b -value) and levels of significance (p-value) (bolded if $p < 0.05$).
Abbreviations are: HbA1c, hemoglobin A1c; R^2, multiple coefficient of determination.

Influence of zinc on serum calcification propensity

To examine whether zinc directly increases T_{50}, zinc was added in the serum calcification propensity assay. Addition of exogenous $ZnCl_2$ significantly modified the T_{50} level in pooled serum from healthy subjects (0 μM, 347 ± 0.8 min; 10 μM, 357 ± 5.6; and 20 μM min, 379.5 ± 4.2 min; $p < 0.001$, Fig. 2A), and pooled serum from dialysis patients (0 μM, 156 ± 2.3 min; 10 μM, 163 ± 0.5 min; and 20 μM, 170 ± 0.8 min, $p < 0.001$, Fig. 2B), respectively.

Discussion

In the present study, we examined the association between serum zinc levels and serum calcification propensity in patients with type 2 diabetes mellitus. Serum zinc level was significantly and positively correlated with T_{50} in the present study. The positive correlation between serum zinc level and T_{50} was also shown in the previous study including healthy subjects and patients with chronic kidney disease [25], indicating this correlation is common in various populations. However, so far, whether serum zinc level could be the independent factor associated with serum T_{50} were not examined. We showed that serum

zinc level was positively associated with T_{50} independent of calcium, phosphate, and magnesium. These novel findings suggest that zinc has an important role in suppressing calcification propensity in serum.

We also confirmed that addition of zinc increases T_{50}, *in vitro* assay. The mechanisms underlying zinc inhibits serum calcification propensity were still unclear. Even in the polyethylene glycol hydrogels, not in serum, zinc inhibits transformation from amorphous calcium phosphate (ACP) into hydroxyapatite [33]. In additive -free composite, ACP transformed into brushite within minutes. In contrast, in the presence of zinc, zinc-doped ACP was very stable and did not show any signs of crystallization for up to 20 days. In ACP, zinc iron readily substitutes calcium [34], suppressing crystallization by decreasing solubility [35]. It is thus likely that zinc suppresses the transformation from amorphous primary CPPs into secondary CPPs, containing crystalline hydroxyapatite, in serum.

In a recent study by Voelkl et al, addition of exogenous $ZnCl_2$ (15 μM) did not improve T_{50} in sera from healthy controls and patients on hemodialysis, although serum zinc level was significantly correlated with T_{50} in those subjects [25]. The discrepancy between the studies by Voelkl et al by us may be explained by difference in $ZnCl_2$ concentration. In the present study, we demonstrated that 10 μM $ZnCl_2$ (= 60.5 μg/dL) did not significantly modify T_{50} in serum from hemodialysis patients, which was consistent with the study by Voelkl et al [25]. In contrast, $ZnCl_2$ concentration of 20 μM (= 131 μg/dL), the upper limit of reference range, could significantly increase T_{50} in those subjects. The crystallization inhibition has been reported to be dependent on the zinc concentration in polyethylene glycol hydrogels [33]. Thus, a certain zinc concentration may be required to increase serum calcification propensity.

In the present study, magnesium was also significantly and positively associated with T_{50}. Magnesium is one of the known anti-calcifying factors, which improves T_{50} in ex vivo [15]. In vitro, magnesium has been reported to prevent phosphate-induced calcification in human aortic VSMC [36]. Similarly, zinc could increase zinc finger protein TNF-α-induced protein 3 (TNFAIP3) expression, which subsequently inhibits NF-kB activation and osteo-/chondrogenic reprograming, resulting suppression of phosphate-induced VSMC calcification [25]. The findings of zinc on T_{50} in the present study, and the above *in vitro* effects of zinc on phosphate-induced calcification in VSMC were similar to those of magnesium. In addition, recently, a randomized control trial has shown that magnesium supplementation increased T_{50} in patients with chronic kidney disease stage 3–4 [37]. Thus, supplementation of zinc, as well as magnesium, might be a potential therapeutic option to attenuate serum calcification propensity and the progression of vasculature calcification. Clearly, however, randomized clinical trials are needed, before such a treatment is recommended.

Albumin is also the anti-calcifying factors associated with T_{50} in ex vivo [15]. When zinc and albumin are included simultaneously in multiple regression analysis, the significant associations of both factors with T_{50} turned to be non-significant (data not shown). Serum zinc acts as an extracellular zinc buffer that controls zinc concentration in blood, since approximately 75–80% of zinc is bound to albumin, accounting for as much as 98% of the exchangeable fraction of zinc in blood [38, 39]. Serum albumin

was significantly and positively correlated with serum zinc levels in the study, thus the confounding effect might explain the results. Measurement of free-zinc irons will be needed to address this issue.

The present study has several limitations. First, the number of subjects examined was relatively small. Second, we cannot be sure whether the findings of this study are applicable to non-diabetic patients. And third, due to the cross-sectional design, we can demonstrate only association, not causality. To confirm the potential benefits of zinc supplementation, further interventional studies are required.

Conclusions

In summary, this is the first report to investigate the association between serum calcification propensity and zinc levels in patients with type 2 diabetes mellitus. Serum zinc was found as an independent factor associated positively with T_{50}, and zinc has an *in vitro* effect on overall propensity of calcification in serum.

Abbreviations

$CaCl_2$: Calcium chloride

Ca × P: calcium-phosphorus product.

CPPs: calciprotein particles.

eGFR: estimated glomerular filtration.

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

NaCl: Sodium chloride

T_{50}: serum calcification propensity.

TNFAIP3: TNF-α-induced protein 3.

VSMCs: vascular smooth muscle cells.

$ZnCl_2$: zinc chloride.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Osaka City University Graduate School of Medicine (approval No. 4100). Opt-out option for informed consent was performed as explained in instructions posted on the website of the institution.

Consent for publication

Not applicable.

Competing Interests

The authors declare that they have no conflicts of interest regarding this study.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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S.N., K.M., and TS. contributed to the concept, design, analysis, interpretation, and writing. M.S., and K.N. contributed to data acquisition and interpretation. H.U., A.T., and M.E. contributed to the concept and interpretation. All authors have read and approved the submitted manuscript.

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Additional information

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Figures

Figure 1

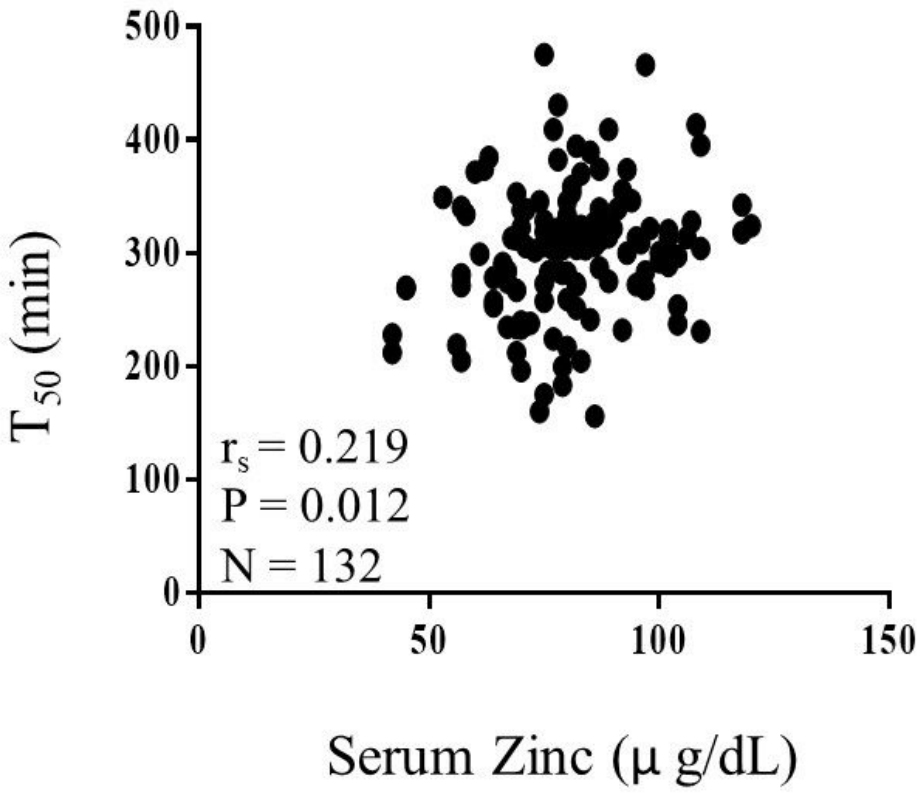

Figure 1

Correlation between serum zinc and serum calcification propensity (T50). Serum zinc correlated positively with T50. Abbreviations: rs, Spearman's correlation coefficient; P, level of significance.

Figure 2

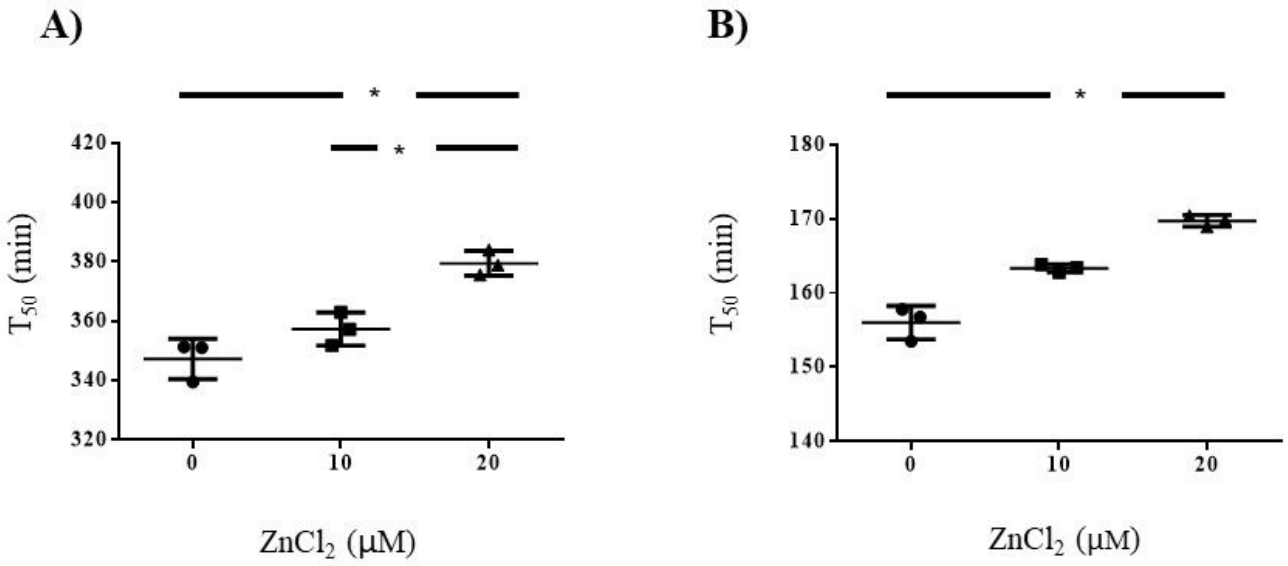

Figure 2

Influence of zinc on serum calcification propensity (T50). Addition of exogenous 20 μM zinc chloride (ZnCl2) significantly increased T50 compared to those of control (0 μM of ZnCl2 addition) in pooled serum from healthy subjects (A), and pooled serum from dialysis patients (B). *p<0.05; statistically significant versus 0 μM ZnCl2 addition. Abbreviations: ZnCl2; zinc chloride