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Original Paper

Partial Reversal of Tissue Calcification and Extension of Life Span following Ammonium Nitrate Treatment of Klotho-Deficient Mice

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Key Words
Calcification • Aging • Life span • Acidosis • Chronic kidney disease • Klotho • $1,25(OH)_2D_3$ • Phosphate • Calcium

Abstract
Background/Aims: Klotho is required for the inhibitory effect of FGF23 on $1,25(OH)_2D_3$ formation and Klotho-hypomorphic mice (*kl/kl*) suffer from severe tissue calcification due to excessive $1,25(OH)_2D_3$ formation with subsequent increase of Ca^{2+} and phosphate concentrations and stimulation of osteogenic signaling. The excessive tissue calcification dramatically accelerates aging and leads to premature death of the animals. Osteogenic signaling in those mice is disrupted by treatment with NH_4Cl, which prevents tissue calcification and early death of *kl/kl* mice. The present study explored whether the beneficial effects of NH_4Cl treatment could be mimicked by NH_4NO_3 treatment. ***Methods:*** The *kl/kl* mice had free access to tap water either without or with addition of NH_4NO_3 (0.28 M) starting with the mating of the parental generation. Calcification of trachea, lung, kidney, stomach, heart and vessels was visualized by histology with von Kossa staining. Plasma phosphate concentration was determined utilizing photometry, blood gas and electrolytes utilizing a blood Gas and Chemistry Analysis System and plasma $1,25(OH)_2D_3$ concentration with ELISA. ***Results:*** In untreated *kl/kl* mice plasma $1,25(OH)_2D_3$ and phosphate concentrations were elevated, and the mice suffered from marked calcification of all tissues analyzed. Untreated *kl/kl* mice further suffered from respiratory acidosis due to marked lung emphysema. NH_4NO_3-treatment decreased both, blood pCO_2 and HCO_3^-, decreased calcification of trachea, lung, kidney, stomach, heart and vessels and increased the life span of *kl/kl* mice more than 1.7-fold (♂) or 1.6-fold (♀) without significantly affecting extracellular pH or plasma

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concentrations of $1,25(OH)_2D_3$, Ca^{2+}, phosphate, Na^+, and K^+. ***Conclusions:*** NH_4NO_3-treatment turns respiratory acidosis into metabolic acidosis and mitigates calcification thus leading to a substantial extension of *kl/kl* mice survival.

Introduction

The protein klotho is required for the inhibitory effect of FGF23 on 25-hydroxyvitamin D_3 1-α-hydroxylase (1-α-hydroxylase) and thus 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) production [1]. Klotho may further up-regulate renal epithelial Ca^{2+} channels [2] and down-regulate renal tubular phosphate transport [3]. Klotho-hypomorphic mice (*kl/kl*) suffer from severe tissue calcification leading to profound growth deficit, premature appearance of several age related disorders and dramatic shortening of life span [1, 4]. Conversely, murine life span is extended by klotho overexpression [5]. In humans, klotho gene variants similarly impact on ageing and life span [6].

Chronic kidney disease (CKD) similarly leads to tissue calcification [7] with negative impact on life span [8]. In CKD tissue calcification is primarily a consequence of impaired phosphate excretion but is compounded by klotho deficiency [9] and affected by a klotho gene variant [10]. Vascular calcification is driven by an active pathophysiological process [11] with transition of vascular smooth muscle cells into an osteo- and chondrogenic phenotypes [12]. Osteogenic reprogramming is stimulated by hyperphosphatemia [13] and hyperaldosteronism [14].

CKD patients and *kl/kl* mice further suffer from acidosis [15, 16], which may counteract $CaHPO_4$ precipitation [17, 18]. Along those lines, tissue calcification of *kl/kl* mice could be prevented by induction of acidosis with acetazolamide [18]. In theory the acidosis could be aggravated by NH_4^+ intake [19, 20]. NH_4^+ may further dissociate to H^+ and the cell membrane permeable NH_3, which enters acidic compartments [21], binds H^+ and is thus trapped as NH_4^+ in those compartments [22] H^+ binding of NH_3 is followed by alkalinization of the acidic cellular compartments [23].

Treatment of *kl/kl* mice with NH_4Cl halts tissue calcification and thus leads to reversal of growth deficit and marked increase of life span [24], effects attributed in large part to disruption of osteogenic signaling due to alkalinization of acidic cellular compartments [24]. The present study explored whether NH_4NO_3 is similarly able to favorably influence tissue calcification and survival of *kl/kl* mice.

Materials and methods

Mice

All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities. Male and female klotho-hypomorphic mice (*kl/kl*) were compared to male and female wild-type mice (WT). The origin of the mice, breeding and genotyping were described previously [4]. The mice had access to either tap water or a solution of NH_4NO_3 (0.28 M) in tap water ad libitum and were fed a standard chow diet (Sniff, Soest, Germany). The NH_4NO_3 treatment started with the mating of the parental generation and was maintained from pregnancy and weaning until analysis of the mice. The offspring was separated from the mothers at the age of four weeks.

Blood and urinary chemistry

To obtain blood specimens, animals were lightly anesthetized and about 50 - 200 µl of blood was withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. The plasma phosphate concentrations were determined utilizing a photometric method (FUJI FDC 3500i, Sysmex, Norsted, Germany). Blood gas and electrolyte analysis as well as the measurement of ionized calcium was performed with the EDAN i15 Blood Gas and Chemistry Analysis System (EDAN Instruments, Shenzen, China). An ELISA kit was employed to determine plasma 1,25(OH)-vitamin D_3 concentration (IDS, Boldon, UK) [25].

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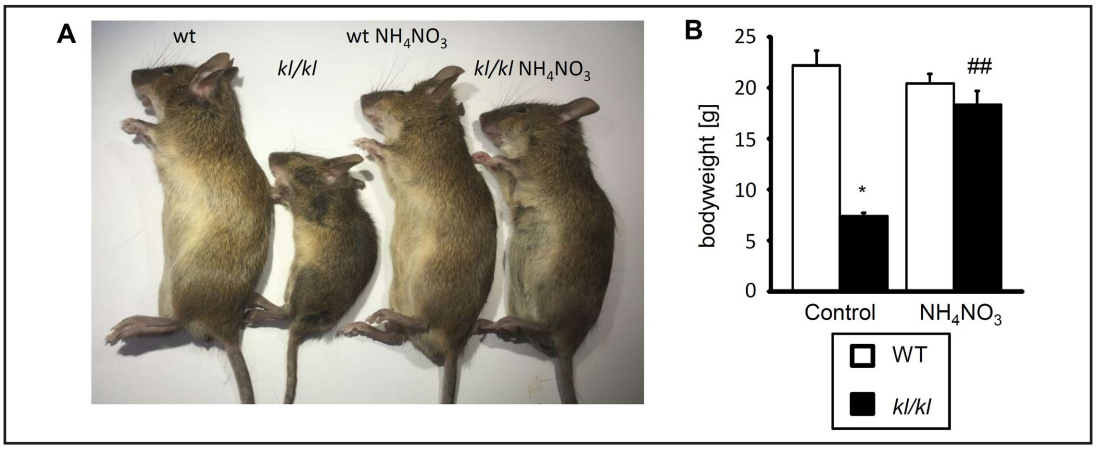

Fig. 1. Effect of NH_4NO_3 treatment on body weight of kl/kl mice. A. Photograph of wild-type mice (*WT*) as well as klotho-hypomorphic mice (*kl/kl*) without (left) and with (right) NH_4NO_3 treatment (0.28 M in tap water). B. Arithmetic means ± SEM (n = 5-9) of body weight of wild-type mice (*WT*, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars) without (Control, left bars) and with (NH_4NO_3, right bars) NH_4NO_3 treatment (0.28 M in tap water). *($p < 0.05$) indicates statistically significant differences from respective WT mice; ##($p < 0.01$) indicates statistically significant differences from respective untreated mice.

Histology

For histological analysis of trachea, lung, kidney, stomach, heart and vessels, tissues from male *kl/kl* mice (age 8 weeks) with or without treatment with NH_4NO_3 (0.28 M in drinking water) were embedded in paraffin, cut in 2–3 µm sections and stained with hematoxylin and eosin (H&E) and von Kossa [26].

Statistics

Data are provided as means ± SEM, *n* represents the number of independent experiments. All data were tested for significance using ANOVA followed by posthoc analysis. For the life span experiments, SAS Jmp version 8.0.1 (SAS Institute Inc., Cary, NC, USA) was used. Only results with $p < 0.05$ were considered statistically significant.

Results

The present study explored whether NH_4NO_3 treatment (0.28 M in tap water) influenced growth deficit, tissue calcification and survival of klotho-hypomorphic (*kl/kl*) mice. As shown in Fig. 1A, *kl/kl* mice were markedly smaller than corresponding wild-type mice (*WT*). Accordingly, the body weight was significantly less in *kl/kl* mice than in wild-type mice (Fig. 1B). Following NH_4NO_3 treatment (0.28 M in tap water), the body weight of *kl/kl* mice was significantly increased. Following NH_4NO_3 treatment the body weight was thus not significantly different between *kl/kl* mice and wild-type mice.

As illustrated in Fig, 2A, plasma $1,25(OH)_2D_3$ concentration was significantly higher in *kl/kl* mice than in wild-type mice, a difference not significantly affected by NH_4NO_3 treatment. Similarly, plasma phosphate concentration was significantly higher in *kl/kl* mice than in wild-type mice, a difference again not significantly affected by treatment with NH_4NO_3 (Fig. 2B). Blood ionized Ca^{2+} was again significantly higher in untreated *kl/kl* mice than in untreated wild-type mice, a difference again not significantly modified by NH_4NO_3 treatment (Fig. 2C). There were no differences in blood Na^+ levels (Fig. 2D) between *kl/kl* mice and wild-type mice and between untreated and NH_4NO_3 treated animals. Plasma K^+ concentration was significantly lower in untreated *kl/kl* mice than in untreated wild-type mice (Fig. 2E), a difference abolished

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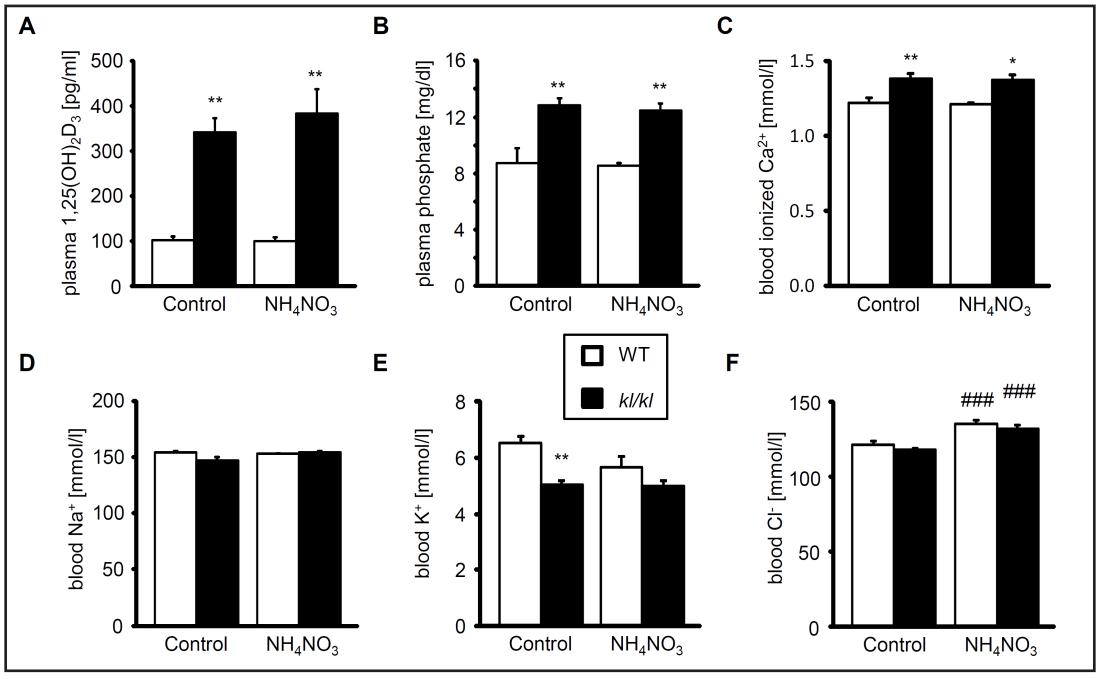

Fig. 2. Plasma $1,25(OH)_2D_3$, phosphate, Ca^{2+}, Na^+, K^+, and Cl^- concentrations of wild-type mice and kl/kl mice with or without NH_4NO_3 treatment. Arithmetic means ± SEM of plasma (A) $1,25(OH)_2D_3$ (n = 7), (B) phosphate (n = 4-9), (C) ionized Ca^{2+} (n = 10), (D) Na^+ (n = 10), (E) K^+ (n = 10), and (F) Cl^- (n = 10) concentrations of wild-type mice (*WT*, white bars) and klotho-hypomorphic mice (*kl/kl,* black bars) without (Control, left bars) and with (NH_4NO_3, right bars) NH_4NO_3 treatment (0.28 M in tap water). *($p < 0.05$), **($p < 0.01$) indicate statistically significant differences from respective WT mice; ###($p < 0.001$) indicate statistically significant differences from respective untreated mice.

by NH_4NO_3 treatment. There were no differences in blood Cl^- concentrations between *kl/kl* mice and wild-type mice (Fig. 2F). NH_4NO_3 treatment significantly increased blood Cl^- concentrations in both, *kl/kl* mice and wild-type mice (Fig. 2F).

As illustrated in Fig. 3A, blood pH was lower in untreated *kl/kl* mice than in untreated wild-type mice. NH_4NO_3 treatment significantly decreased blood pH in wild-type mice. NH_4NO_3 treatment tended to decrease blood pH in *kl/kl* mice, an effect, however, not reaching statistical significance (Fig. 3A). As shown in Fig. 3B, blood pCO_2 was significantly increased in *kl/kl* mice and significantly decreased to normal values under treatment with NH_4NO_3. Plasma HCO_3^- concentration was significantly higher in untreated *kl/kl* mice than in untreated wild-type mice, a difference again significantly blunted by NH_4NO_3 treatment (Fig. 3 C,D).

Histology was employed to visualize tissue calcification and histopathology in untreated and NH_4NO_3 treated male *kl/kl* mice. As illustrated in Fig. 4, excessive calcification was observed in trachea, lung, kidney, and stomach of *kl/kl* mice. Moreover, histology revealed marked emphysema of lung tissue from *kl/kl* mice. NH_4NO_3 treatment strongly reduced the tissue calcification and reversed the emphysema of *kl/kl* mice. As shown in Fig. 5, excessive calcifications were similarly observed in cardiac and vascular tissue of untreated *kl/kl* mice. Again, NH_4NO_3 treatment blunted the calcification of cardiac and vascular tissue from *kl/kl* mice.

As illustrated in Fig, 6, NH_4NO_3 treatment was followed by a substantial and significant (Log-Rang $p < 0.0004$; Wilcoxon $p < 0.0016$) increase of the life span of male *kl/kl* mice. Half of untreated *kl/kl* mice survived 72 days and half of the NH_4NO_3 treated *kl/kl* mice survived 122 days. Accordingly, NH_4NO_3 treatment extended the median life span of *kl/kl* mice by a factor of 1.7.

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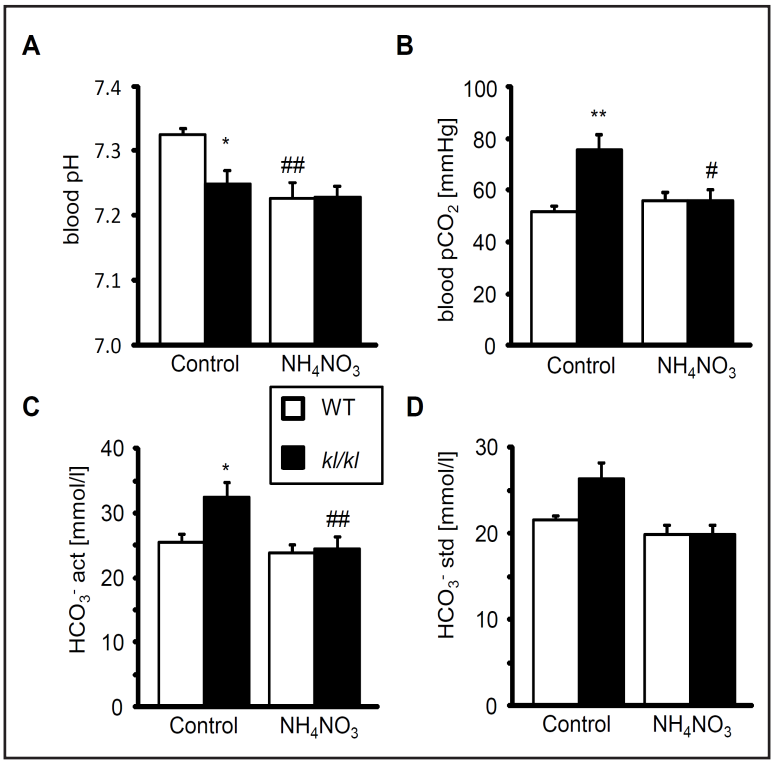

Fig. 3. Blood pH, pCO_2, HCO_3^- act. and HCO_3^- std. concentrations of wild-type mice and kl/kl mice with or without NH_4NO_3 treatment. Arithmetic means ± SEM (n = 10) of blood (A) pH, (B) pCO_2, (C) actual HCO_3^- and (D) HCO_3^- standardized to normal CO_2, in wild-type mice (*WT*, white bars) and klotho-hypomorphic mice (*kl/kl*, black bars) without (Control, left bars) and with (NH_4NO_3, right bars) NH_4NO_3 treatment (0.28 M in tap water). *($p < 0.05$), **($p < 0.01$) indicate statistically significant differences from respective *WT* mice; #($p < 0.05$), ##($p < 0.01$) indicate statistically significant differences from respective untreated mice.

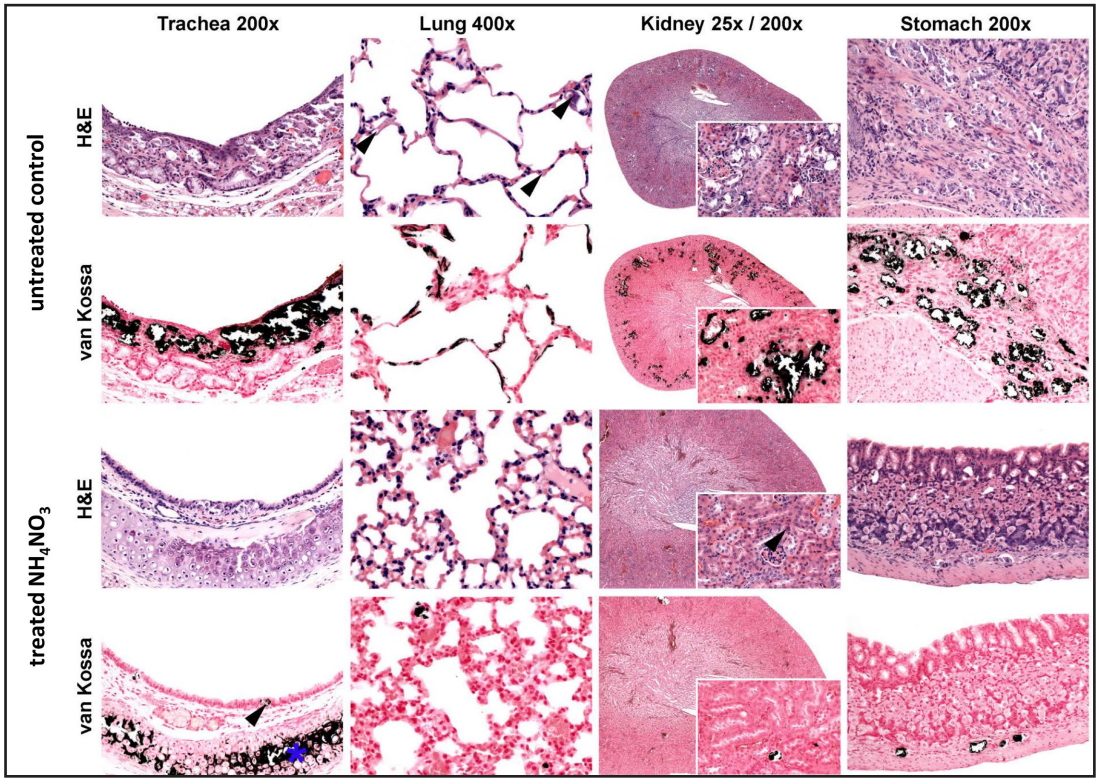

Fig. 4. NH_4NO_3 treatment counteracts soft tissue calcification in kl/kl mice. H&E and von Kossa staining of trachea, lung, kidney, and stomach from male klotho-hypomorphic mice without (untreated, upper panel) and with (treated, lower panel) NH_4NO_3 treatment (0.28 M in tap water). The results are representative for three *kl/kl* mice per group. Arrowheads indicate the calcifications; Asterisk indicates the cartilage.

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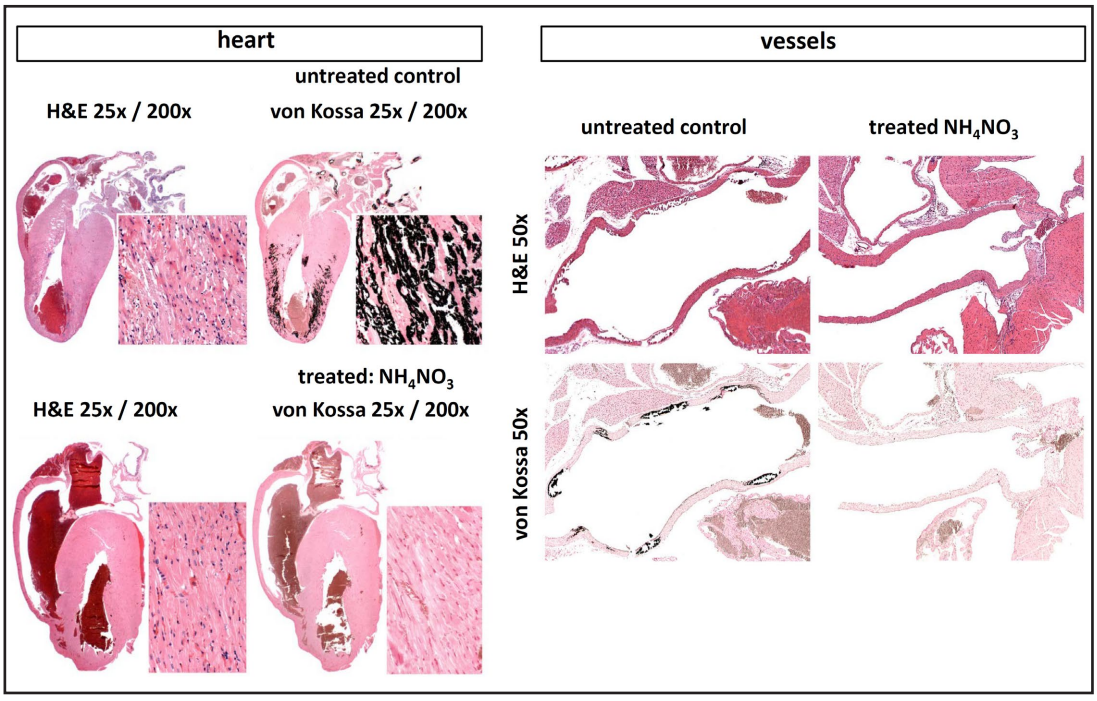

Fig. 5. NH_4NO_3 treatment counteracts cardiac and vascular calcification in kl/kl mice. H&E and von Kossa staining of cardiac and vascular tissue from male klotho-hypomorphic mice without (untreated, upper panel) and with (treated, lower panel) NH_4NO_3 treatment (0.28 M in tap water). The results are representative for three *kl/kl* mice per group.

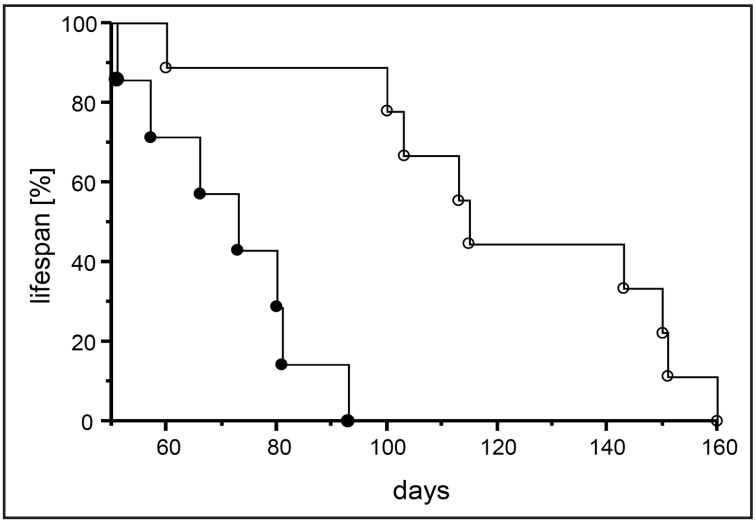

Fig. 6. Effect of NH_4NO_3 on life span of kl/kl mice. Percentage of surviving male klotho-hypomorphic mice (*kl/kl*) maintained on control diet without treatment (closed circles) and with NH_4NO_3 treatment (0.28 M in tap water, open circles) as a function of age. Survival of *kl/kl* mice was significantly extended by NH_4NO_3 treatment (Log-Rang ($p < 0.0004$, Wilcoxon $p < 0.0016$; $n = 7 - 9$).

Discussion

The present study confirms the phenotype of *kl/kl* mice, i.e. the severe tissue calcification, growth deficit and accelerated aging presumably resulting from unrestricted formation of $1,25(OH)_2D_3$ with subsequent marked increase of Ca^{2+} and phosphate concentrations leading to triggering of osteogenic signaling [1]. The present study further reveals that NH_4NO_3 treatment mitigates tissue calcification of *kl/kl* mice, leading to almost complete reversal of growth deficit, attenuation of tissue injury and substantial extension of life span. All those

effects mimic those of NH_4Cl treatment, which has previously been shown to disrupt the excessive osteogenic signaling of *kl/kl* mice [24].

The tissue calcification of *kl/kl* mice is most likely due to excessive extracellular phosphate concentrations, which are known to stimulate osteogenic signaling and vascular calcification [13], which is in turn a hallmark of aging [7, 27-29]. Along those lines plasma phosphate concentration is a determinant of mortality [30]. NH_4NO_3 treatment mitigates tissue calcification in *kl/kl* mice without significantly modifying plasma $1,25(OH)_2D_3$, Ca^{2+} and phosphate concentrations. NH_4NO_3 treatment is further effective despite virtually constant extracellular pH, another determinant of calcification [18, 31-33].

NH_4NO_3 treatment does not aggravate the acidosis of *kl/kl* mice, which is due to excessive pCO_2 presumably due to the severe lung emphysema (Fig. 4). The NH_4NO_3 treatment reverses the hypercapnia presumably by counteracting the development of lung emphysema (Fig. 4). At the same time, however, NH_4NO_3 treatment decreases plasma bicarbonate concentration, presumably due to partial incorporation of NH_4^+ into urea, a metabolic pathway consuming HCO_3^- [34]. Following NH_4NO_3 treatment bicarbonate and pCO_2 decline in parallel leaving extracellular pH virtually unchanged. Accordingly, NH_4NO_3 treatment converts the respiratory acidosis of untreated *kl/kl* mice into a metabolic acidosis.

Similar to the excessive osteogenic signaling in *kl/kl* mice [24, 35], enhanced osteogenic signaling leads to vascular calcification in CKD patients [35, 36]. In CKD vascular calcification increases the risk for cardiovascular events [37], the leading cause of death in this clinical condition [38]. Similar to the osteogenic signaling in *kl/kl* mice, osteogenic signaling in CKD patients [39] is secondary to hyperphosphatemia and is compounded by decrease of klotho expression [35]. Disruption of osteogenic reprogramming in vascular tissue is thus expected to favourably influence the clinical course of CKD [39].

Conclusion

Treatment with NH_4NO_3 decreases tissue and vascular calcification, reverses the growth deficit and substantially extends the life span of klotho-hypomorphic mice despite continued increase of plasma $1,25(OH)_2D_3$, Ca^{2+} and phosphate concentrations. In view of the previous observations following NH_4Cl treatment, NH_4NO_3 treatment is presumably effective by disrupting osteogenic signaling.

Disclosure Statement

The authors state that they do not have any conflicts of interest to disclose.

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