Calcitriol Exerts Prophylactic Anti-Mycobacterium Effect In A Dose-Dependent Manner

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Abstract

Vitamin D was empirically applied for Tuberculosis (TB) treatment in the past, and is currently used as an adjuvant for TB therapy. Although an increasing pile of evidences suggests that vitamin D has no therapeutic effect against TB infection, the prophylactic effect of vitamin D in preventing TB remains largely undetermined. To experimentally valuate the potential prophylactic effect of calcitriol (the active form of vitamin D) against mycobacterium infection, we performed dose-gradient calcitriol soaking in 30-day-old zebrafish before Mycobacterium marinum (*M. marinum*) challenge through tail vein injection. 1H-NMR metabolomics analysis was further performed for illustration of potential mechanisms underlying the prophylactic effect of calcitriol against *M. marinum*. The results suggested that calcitriol exerts dose-dependent prophylactic anti-mycobacterium effects, i.e., the bacterial load and the corresponding inflammatory factors (IL-1β, TNF-α, and IFN-γ) expressions in *M. marinum* challenged zebrafish were reduced by low-dose (25 µg/L) or high-dose (2500 µg/L) calcitriol soaking, rather than by moderate-dose (250 µg/L) calcitriol soaking. Body weight of the *M. marinum* challenged zebrafish was recovered by high-dose prophylactic calcitriol soaking rather than by low-dose or moderate-dose calcitriol. The 1H-NMR metabolomic profiling identified 29 metabolites with altered abundance among the dose-gradient calcitriol groups, among which 22 metabolites were co-varied with the dose of calcitriol, the rest 7 metabolites were co-varied with the bacterial load and the inflammatory response in term of cytokine expression. Further pathway analysis indicated that the glycine, serine, and threonine metabolism pathway was the activated in both of the two metabolite groups, indicating that the pathway was altered by dose-gradient of calcitriol and was in response to *M. marinum* infection in zebrafish. The results of the present study suggested that the activation of glycine, serine and threonine metabolism pathway may play a potential role for the dose-dependent anti-mycobacterium effect induced by prophylactic calcitriol soaking.

Introduction

Vitamin D, with calcitriol (1α,25-Dihydroxyvitamin D3) as its major active form, is well known for calcium homeostasis[1, 2]. Deficiency of vitamin D has been linked with establishing tuberculosis (TB)[3–5], and is highly prevalent in pulmonary TB patients[6, 7]. Supplementation of Vitamin D in the medication of pulmonary TB is thus believed to be efficacious and extensively popularized especially before the application of antibiotics in controlling TB[8, 9]. However, results of recent large-cohort trials did not support the effectiveness of Vitamin D in TB treatment[10–14]. Similar outcomes were obtained between Vitamin D and the placebo, suggesting limited effects of Vitamin D on TB treatment[15, 16].

Although the therapeutic effect of Vitamin D on TB is suspicious, its prophylactic anti-TB effect and the underlying mechanism remain largely unknown. Meanwhile, dozens of researches have reported the dose-dependent effects of Vitamin D on oxidative stress and apoptosis[17], immunomodulation[18], volumetric bone density and bone strength[19], as well as trans-differentiation of muscle to adipose cells[20]. Whether Vitamin D effectively exert a dose-dependent prophylactic anti-TB effect is undetermined.
To experimentally valuate the potential prophylactic anti-mycobacterium effect of Vitamin D, we performed dose gradient calcitriol soaking in adult zebrafish before mycobacterium marinum (*M. marinum*) challenge. 1H-NMR metabolomics analysis was further applied to reveal the pathways activated in *M. marinum* infected zebrafish receiving prophylactic calcitriol soaking.

**Materials And Methods**

**Study design and Calcitriol soaking.**

AB strain zebrafish (Danio rerio) was bought from China Zebrafish Resource Center. Embryos were collected from natural crosses and raised in E3 media (0.33 mM MgSO4 7H2O, 0.33mM CaCl2 2H2O, 0.17mM KCl, 5mM NaCl), with a 14h light/10h dark cycle at 28.5°C. One-month old (30 days post fertilization, 30 dpf) zebrafish was raised under a soaking media of calcitriol (25, 250, 2500 µg/L) in E3 media with a replacement frequency of 12h for one month. The *M. marinum* strain ATCC 927 was injected to the 2-month-old (60 dpf) zebrafish through tail vein with a dose of 100 CFU/fish. The *M. marinum* infected zebrafish was further raised for half a month and suicided at 75 dpf. The suicided fishes were weighted and stored in a -80°C deep freezer. A detailed schematic diagram of the experimental design was shown in Fig. 1a.

**Total nucleic acids extraction and quantification of bacterial load and inflammatory factors**

The freezed fish was grinded with liquid nitrogen. Total nucleic acids (DNA and RNA) were then extracted by a bead-based DNA/RNA co-extraction kit (Cat No. DP438, TIANGEN Biotech, Beijing, China) according to the manufacture's instructions. The extracted total nucleic acids were further applied for quantification of bacterial load and inflammatory factors. Total nucleic acids from a known amount of the cultured *M. marinum* and the 10-fold dilutions were extracted with the same method as templates for generation of absolute qPCR standard curve of bacterial load.

The bacterial load was quantified by absolute qPCR using TB Green Fast qPCR Mix (Takara Biotechnology (Dalian), China) and a primer set targeting 16s V3-V5 region (Forward: 5’-CACACGAGAACACTCCAA-3’, Reverse: 5’-ACATCCCGAAACCAACAGAG-3’). Each 25 µl reaction mix contained 12.5 µl TB Green Fast qPCR Mix, 0.4 µM each primer, 2 µl total nucleic acids, 8.5 µl nuclease-free water. qPCR amplifications were carried out by using Bio-Rad CFX96 (Bio-Rad, CA, USA) with the following thermo-cycling conditions: 30 sec at 95°C for pre-denaturation, followed by 40 cycles of 5 sec at 95°C and 10 sec at 60°C. Fluorescence measurements were taken at 60°C of each cycle. The threshold cycle (Ct) value was defined as the point at which the fluorescence signal exceeded the means plus 10 standard deviations of the baseline. A standard curve between log10 CFU of the 10-fold diluted *M. marinum* and the corresponding Ct values were established by using Microsoft Excel 2010. The bacterial load of each fish was determined by applying the Ct value to the above-mentioned standard curve.
Relative quantification of three major inflammatory factors (IFN-γ, IL-1β, TNF-α) were performed by using HiScript II One Step qRT-PCR SYBR Green Kit (Vazyme Biotech, Nanjing, China). The primer sets are as follows: IFN-γ (Forward: 5'- CCG ATACA GGATAATAA CGA CAG − 3', Reverse: 5'- GACCCTTGCCTTGGCTTGCGATGA − 3'); IL-1β (Forward: 5'- TGGTGATTCCATGCGCCTTAC − 3', Reverse: 5'- CCACCATCTGCGAATCTTCATAC − 3'); TNF-α (Forward: 5'- CTTTACCGCTGGATGCTGTCC − 3', Reverse: 5'- AAAGACACCTGGCTGTAGACAAA − 3'). Each 20 µl reaction mix contained 10 µl 2×One Step SYBR Green Mix, 1 µl One Step SYBR Green Enzyme Mix, 0.4 µl each 10 µM primer, 2 µl total nucleic acids, 6.2 µl nuclease-free water. qRT-PCR amplifications were carried out with the following thermocycling conditions: 3min at 50°C for reverse transcription, 30 sec at 95°C for pre-denaturation, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Fluorescence measurements were taken at 60°C of each cycle. Using the house-keeping gene GAPDH as an internal control, the relative expression of three inflammatory factors were calculated using the 2^−ΔΔCT method.

1H-NMR metabolomics

Fish sample preparation for 1H-NMR metabolomics was performed according to a previously reported procedure with some modifications[21]. Briefly, one fish was mixed with 1 ml D2O containing 0.05% TSP (3-trimethylsilyl-[2,2,3,3-D4]-propionate, as internal standard), and homogenized in an ice-water bath with the IKA T10 Basic ULTRA-TURRAX disperser (IKA, Germany). A total of 550 µl supernatant from each sample was obtained through centrifuging of the homogenate at 4°C, 15 000 rpm for 15 min, and transferred into a 5 mm NMR tube for further 1H-NMR spectral profiling. 1H-NMR spectrometry was profiled by using a Bruker 600 MHz AVANCE III NMR spectrometer (Bruker BioSpin, Germany) with a NOESYGPPR1D pulse sequence and the following parameters: spectral width 12345.7 Hz, spectral size 65536 points, pulse width 40.5 µs, relaxation delay 1.0 s, 64 scans. Spectra processing was performed by MestReNova (v8.0.1, Mestrelab Research, Spain) with manually corrected phase and baseline. The chemical shift of TSP was calibrated at 0.00ppm and the spectral region of δ 0.16–9.58 was segmented at 0.01 ppm width after exclusion of the region of residual water δ 4.60–5.20. The obtained NMR data was normalized to the total sum of spectra, log-transformed and auto scaled (mean-centered and divided by the standard deviation of each NMR feature) before further analysis.

Multivariate statistical analysis was performed by using SIMCA-P (v14.1, Umetrics AB, Sweden). Principle Component Analysis (PCA) was applied for investigation of the natural separation among sample and for exclusion of the outliers using the Hotelling's T^2 statistic. Orthogonal Projection to Latent Structures Discriminant Analysis (OPLS-DA) was performed to interpretate types of variation with between-group discriminating power by incorporating the grouping information. The best-fit OPLS-DA model was validated by a cross-validation of all models using a 200-step permutation test. The parameters R2Y and Q2 were applied for assessment of the fitting validity and the predictive ability of the selected OPLS-DA model, respectively. Metabolites were identified from the 1H-NMR features by searching the human metabolome database (HMDB, https://www.hmdb.ca) and previously published articles with the following parameters: chemical shift, coupling constant, and peak type. Altered metabolites were defined as metabolites with altered between-group abundances, and simultaneously meet the following criteria:
Importance for the Projection (VIP) > 1 in the OPLS-DA model and false discovery rate (fdr)-adjusted $P < 0.05$ in an independent-sample T-test. Metabolic pathway analysis was performed by the pathway analysis module implemented in the web portal of MetaboAnalyst (https://www.metaboanalyst.ca). The Danio rerio (zebrash) (KEGG) library was applied for pathway analysis with the following algorithms: hypergeometric test for over representation analysis, relative-betweenness centrality for pathway topology analysis.

**Statistical analysis**

Between-group statistical analyses (implemented in GraphPad Prism v8.0) were performed by two-tailed paired t-test for body weight and bacterial load, non-parametric Mann-Whitney test for the relative expression of inflammatory factors. $P < 0.05$ was defined as statistically significant for testing of body weight, bacterial load, and relative expression of inflammatory factors. $P$ values from the t-test of 1H-NMR spectra profiling were adjusted by false discovery rate (fdr) with R package vegan for multiple testing correction. The fdr-adjusted $P$ value $< 0.05$ was defined as statistically significant.

**Results**

**Prophylactic calcitriol soaking exerted dose-dependent anti-mycobacterium effects in adult zebrafish**

Calcitriol has been reported to have therapeutic effect against mycobacterium infection[22, 23]. To determine if this effect is prophylactic and dose-dependent, one-month old (30 days post fertilization, dpf) adult zebrafish were firstly treated with calcitriol soaking for one month, then were challenged with *M. marinum* through tail vein injection (at 60 dpf), and were monitored for another half a month (61–75 dpf) (Fig. 1a). At the time point of 75 dpf, the body weight of *M. marinum* challenged zebrafish was significantly decreased (Fig. 1b, $P < 0.001$). Low-dose (25 µg/L) and moderate-dose (250 µg/L) calcitriol soaking has no significant effect to the weight loss induced by *M. marinum*, while high-dose (2500 µg/L) calcitriol soaking markedly recovered the weight loss (Fig. 2a, $P < 0.05$). Corresponding to this finding, the bacterial load in *M. marinum* challenged zebrafish was reduced by high-dose calcitriol soaking (Fig. 1c, $P < 0.0001$), and by low-dose calcitriol soaking (with no statistical significance), but seemed to be not affected by moderate-dose calcitriol soaking (Fig. 1c). These results suggested that prophylactic calcitriol soaking exerts dose-dependent effects on body weight and bacterial load in *M. marinum* challenged adult zebrafish, with significantly recovered weight loss by high-dose calcitriol and reduced the bacterial load by low-/high-dose calcitriol, but both seemed not be affected by moderate-dose calcitriol.

**Prophylactic calcitriol soaking induced dose-dependent anti-inflammatory effects in *M. marinum* infected adult zebrafish**

To further determine the effects of prophylactic calcitriol soaking to the inflammatory level of *M. marinum* challenged zebrafish, we measured the relative expression levels of three major inflammatory factors, including IL-1β, TNF-α and IFN-γ (Figure 1 d-f). Significant elevated IL-1β (Figure 1d, $P < 0.01$) and IFN-γ (Figure 1e, $P < 0.01$) were observed in *M. marinum* challenged zebrafish, while TNF-α was also
elevated (Figure 1f, with no statistical significance comparing to the control group). IFN-γ (Figure 1e) and TNF-α (Figure 1f) were significantly down-regulated by low-dose or high-dose calcitriol, while IL-1β was significantly down-regulated by high-dose calcitriol (Figure 1d, \( P < 0.01 \)) rather than low-dose calcitriol (Figure 1d, \( P > 0.05 \)). Interestingly, moderate-dose calcitriol seemed to have no significant effect on the levels of IFN-γ (Figure 1e, \( P > 0.05 \)) and TNF-α (Figure 1f, \( P > 0.05 \)), and even up-regulated the elevated level of IL-1β (Figure 1d, \( P < 0.05 \)). When comparing among the three doses of calcitriol, moderate-dose calcitriol significantly up-regulated the levels of IL-1β (Figure 1d, \( P < 0.01 \)) and TNF-α (Figure 1f, \( P < 0.01 \)). These results demonstrated that prophylactic calcitriol soaking exerted dose-dependent anti-inflammatory effects in \textit{M. marinum} challenged zebrafish, with low- and high-dose calcitriol significantly down-regulating the elevated inflammatory levels but moderate-dose calcitriol exerting pro-inflammatory effects in an opposite direction.

**Prophylactic calcitriol soaking induced metabolomic shift in \textit{M. marinum} infected adult zebrafish**

Because moderate-dose prophylactic calcitriol soaking exerted pro-inflammatory effects and no marked effect to the weight loss and the bacterial load in \textit{M. marinum} challenged zebrafish in contrast to low- and high-dose calcitriol soaking, we performed 1H-NMR metabolomic profiling to investigate the potential underlying mechanism. Scatter plot of PCA (Fig. 2a) demonstrated that the metabolome of \textit{M. marinum} challenged zebrafish with prophylactic calcitriol soaking were clearly separated, suggesting that metabolomic shifts occurred in response to different dose of calcitriol. To further evaluate the metabolomic shift, we performed OPLS-DA among the \textit{M. marinum} challenged zebrafishes receiving low-/moderate-/high-dose of prophylactic calcitriol soaking. From the scatter plot of OPLS-DA among the three dose groups (Fig. 2b, \( Q^2 = 0.77 \), indicating that the OPLS-DA model explained 77.0% of sample variation), moderate-dose calcitriol group was far away from low-dose and high-dose calcitriol groups along the X-axis (explained 66.7% of the variation in the independent variable X (\( R^2X = 0.677 \))), and clustered together along the Y-axis (explained 83.3% of the variation in the categorical variable Y (\( R^2Y = 0.833 \))). While low-dose group and high-dose calcitriol group was also separated with a relatively high with-in group variation (Y-axis) in high-dose calcitriol group. These results suggested that larger metabolic shift occurred in moderated-dose calcitriol group than in high-dose calcitriol group of \textit{M. marinum} challenged zebrafish.

To determine the metabolites contributing to the metabolomic shift among different dose of calcitriol, we performed pair-wised OPLS-DA (Fig. 2c, e, g validated by 200-step permutation tests in Figure S1 a-c) and S-plot analysis (Fig. 2d, f, h). A total of 895 1H-NMR features were profiled, from which 692 features were retained after removal of features with minus relative abundances in over half of the sample. Eight one features with altered between-group abundances among the three groups of calcitriol soaking were obtained, which met the criteria that VIP \( \geq 1 \) and \textit{fdr-adjusted} \( P < 0.05 \) in an independent t-test. Totally 29 metabolites from the 81 features with altered between-group abundances were structurally identified (Table 1), among which the abundances of 22 metabolites (Fig. 3) were down-regulated and the abundance of methylguanidine was up-regulated from low-dose across moderate-dose to high dose calcitriol, the abundances of 7 metabolites were up-regulated by moderate-dose calcitriol but down-
regulated by high-dose calcitriol (Fig. 3, marked with an asterisk behind the metabolite name). These results suggested that a larger part of 22 metabolites with altered abundances were strictly in response to dose-gradient prophylactic calcitriol soaking, while a smaller part of 7 metabolites were co-varied with the bacterial load and inflammatory factors.
| Metabolite                  | Chemical shift                  | VIP   | p[1]   | p(corr)[1] | P-Value | fdr-adjusted P-Value |
|-----------------------------|---------------------------------|-------|--------|------------|---------|---------------------|
| N-Acetyl-L-alanine          | 1.315d, 2.000s                   | 2.18  | -0.09  | -0.87      | 5.31E-03| 9.21E-03            |
| Palmitoylcarnitine          | 2.69d, 3.20s, 3.70d, 3.83m, 5.58dt | 1.02  | -0.04  | -0.76      | 2.79E-02| 3.02E-02            |
| Putrescine                  | 1.754m, 3.040t                   | 3.83  | -0.16  | -0.78      | 1.69E-02| 2.09E-02            |
| L-Histidine                 | 3.16dd, 3.98dd, 7.09d, 7.90d     | 1.13  | -0.05  | -0.74      | 4.87E-03| 8.74E-03            |
| D-Glucuronic acid           | 3.290t, 4.085d, 4.649d, 5.250d   | 4.17  | -0.17  | -0.93      | 1.53E-03| 5.89E-03            |
| Methylguanidine             | 2.833s, 3.366s                   | 17.60 | 0.72   | 0.84       | 7.58E-03| 1.23E-02            |
| Taurine                     | 3.250t, 3.417t                   | 2.88  | -0.12  | -0.90      | 2.91E-03| 7.30E-03            |
| 3-Methylxanthine            | 3.510s, 8.020s                   | 1.74  | -0.07  | -0.76      | 2.59E-02| 2.87E-02            |
| L-Threonine                 | 1.316d, 3.575d                   | 1.48  | -0.06  | -0.82      | 2.95E-03| 7.30E-03            |
| N-Acetylneuraminic acid     | 2.009m, 2.045s, 3.718m, 3.752t, 4.019t | 1.36  | -0.06  | -0.85      | 3.75E-03| 7.50E-03            |
| Mannitol                    | 3.649dd, 3.771d, 3.841dd         | 1.47  | -0.06  | -0.94      | 2.57E-04| 2.70E-03            |
| Dimethylglycine             | 2.910s, 3.710s                   | 1.37  | -0.06  | -0.78      | 1.37E-02| 1.87E-02            |
| Propionylglycine            | 1.113t, 2.300q, 3.745d           | 1.69  | -0.07  | -0.78      | 1.49E-02| 1.93E-02            |
| Guanidoacetic acid          | 3.780s                          | 1.53  | -0.06  | -0.73      | 1.17E-02| 1.69E-02            |
| L-Allothreonine             | 1.195d, 3.835d                   | 1.93  | -0.08  | -0.94      | 6.28E-04| 5.08E-03            |
| N-Acetylgalactosamine       | 2.05d, 3.875d, 3.910d, 3.995d, 4.117m, 5.225d | 1.30  | -0.05  | -0.87      | 1.70E-03| 5.89E-03            |
| Betaine                     | 3.250s, 3.890s                   | 1.83  | -0.08  | -0.81      | 1.84E-02| 2.12E-02            |
| Metabolite               | Chemical shift                        | VIP | p[1]   | p(corr) | P-Value     | fdr-adjusted P-Value |
|-------------------------|---------------------------------------|-----|--------|---------|-------------|----------------------|
| 7-Methylxanthine        | 3.91d, 7.84d                          | 1.90| -0.08  | -0.82   | 8.09E-03    | 1.28E-02             |
| 2-Hydroxybutyric acid  | 0.886t, 1.641m, 3.990dd                | 1.33| -0.05  | -0.79   | 2.53E-03    | 7.30E-03             |
| Caffeine                | 3.410s, 3.590s, 4.000s                 | 1.25| -0.05  | -0.83   | 1.32E-04    | 2.70E-02             |
| FAD                     | 2.32s, 2.39s, 4.06m, 5.82d, 7.54s, 7.85s, 8.30s | 1.58| -0.06  | -0.94   | 5.05E-05    | 2.63E-03             |
| Carnosine               | 2.672q, 3.216t, 7.052s, 8.015s         | 1.62| -0.07  | -0.82   | 1.45E-02    | 1.93E-02             |
| Deoxyuridine            | 3.845dd, 4.052m, 5.895d, 6.287t, 7.850d | 1.62| -0.07  | -0.81   | 3.63E-03    | 7.50E-03             |
| Hypoxanthine            | 8.170s, 8.200s                         | 1.11| -0.05  | -0.79   | 2.69E-03    | 7.30E-03             |
| Tryptamine              | 3.170t, 3.337t, 7.320s, 7.545d, 7.695d | 1.05| -0.05  | -0.69   | 4.11E-02    | 4.90E-02             |
| Choline                 | 3.189s, 4.056m                         | 2.98| -0.12  | -0.65   | 4.15E-02    | 2.99E-01             |
| Glycerophosphocholine   | 3.200s, 3.637m, 4.305m                 | 5.71| -0.23  | -0.89   | 9.30E-03    | 1.49E-01             |
| p-Hydroxyphenylacetic acid | 3.440s, 6.850d, 7.150d              | 2.17| -0.09  | -0.90   | 9.60E-03    | 1.49E-01             |
| Creatine                | 3.020s, 3.920s                         | 2.91| -0.12  | -0.72   | 1.50E-02    | 1.79E-01             |

p[1], covariance coecient calculated with the principle component 1 of the OPLS-DA model. FAD, Flavin adenine dinucleotide.

p(corr)[1], correlation coecient calculated with the principle component 1 of the OPLS-DA model. fdr: false discovery rate.

s: single-peak. d, double peak. t, triplet peak. q, quarter peak. m, multiple peaks.

**Prophylactic calcitriol soaking induced metabolomic shift was associated with its antitycobacterium/anti-inflammatory effects**

Because the altered metabolites contributing to the metabolic shift induced by prophylactic calcitriol soaking in *M. marinum* challenged zebrafish co-varied with the dose-gradients of calcitriol or inflammatory factors, we performed spearman rank correlation analysis to confirm the associations among the altered metabolites, the weight loss, the bacterial load, and the inflammatory factors. The
spearman rank correlation matrix (Figure 4a, Table S1-S2) exhibited that TNF-α was positively correlated with Dimethylglycine ($\rho = 0.60$, $P < 0.01$); the body weight was positively correlated with Methylguanidinium ($\rho = 0.70$, $P < 0.01$) and negatively correlated with L-Allothreonine, Mannitol, N-Acetylgalactosamine, Hypoxanthine, L-Histidine, Creatine, N-Acetylneuraminic acid, Betaine, D-Glucuronic acid, Palmitoyl carnitine, 2-Hydroxybutyric acid, Putrescine, L-threonine, and Guanidoacetic acid ($\rho < -0.532$, $P < 0.01$); the bacterial load was negatively correlated with most of the altered metabolites ($P < 0.05$) with lower correlation coefficient (the absolute $p$ value less than 0.53). Among the altered metabolites, Methylguanidinium was negatively correlated with most metabolites, the above TNF-α/body weight/bacterial load associated metabolites were positively correlated with each other (Figure 4a, Table S1-S2, $\rho > 0.532$, $P < 0.01$). These results demonstrated that the metabolites contributing to the metabolic shift by prophylactic calcitriol soaking in *M. marinum* challenged zebrafish correlated with each other and with the phenotypes.

**Glycine, serine and threonine metabolism was correlated with prophylactic calcitriol soaking and its anti-mycobacterium/anti-inflammatory effects**

To further infer the underlying pathways of the altered metabolites, we performed pathway analysis by using the module implemented in the MetaboAnalyst web portal (Fig. 4b-c). Pathway analysis from the 22 altered metabolites co-varied with the dose-gradients of calcitriol (Fig. 4b) suggested that three pathways were significantly altered, including glycine, serine and threonine metabolism (hypergeometric test, $P = 9.32E-5$), beta-alanine metabolism (hypergeometric test, $P = 0.029$), and histidine metabolism (hypergeometric test, $P = 0.021$). Pathway analysis from the 7 altered metabolites co-varied with bacterial load and inflammatory factors (Fig. 4c) suggested that glycine, serine and threonine metabolism was significantly altered (hypergeometric test, $P = 0.0049$). Glycine, serine, and threonine metabolism was predicted by both group of metabolites, with 4 metabolites (Betaine, L-Threonine, L-Allothreonine, and Guanidoacetic acid, Fig. 4d, name in pink) co-varied with the dose gradients of calcitriol and 3 metabolites (Choline, Dimethylglycine, and Creatine, Fig. 4d, name in blue) co-varied with the bacterial load and the inflammatory factors. These results indicated that alteration in glycine, serine and threonine metabolism was induced by prophylactic calcitriol soaking and was in response to the dose-dependent anti-mycobacterium and anti-inflammatory effects, suggesting a potential role of the pathway in host-microbe interactions.

**Discussion**

Although Vitamin D has been proved to promote macrophage-mediated killing of *Mycobacterium Tuberculosis*, a puzzle still exists in the benefit of Vitamin D in terms of tuberculosis treatment outcomes in light of increasing negative evidences from randomized, double-blind, placebo-controlled trials. Nevertheless, the prophylactic effect rather than the therapeutic effect of Vitamin D in tuberculosis prevention was rarely investigated. Because human trial-based evaluation of the prophylactic effect of Vitamin D is hard to carry out, we applied *M. marinum* challenged zebrafish as an animal model mimicking tuberculosis infection in human to investigate the prophylactic effect of calcitriol (the active
form of Vitamin D) against *M. marinum* infection. We observed dose-dependent anti-mycobacterium and anti-inflammatory effects of calcitriol in *M. marinum* challenged zebrafish. Further metabolomic investigation suggest that the glycine, serine, and threonine metabolism pathway may play a role in the prophylactic effects of calcitriol.

A previous report[24] suggested that the anti-mycobacterium activity of calcitriol might operate at helping alveolar macrophages and tissue dendritic cells in preventing the initial implantation and ingestion of bacilli. Thus, a constant circulating level of calcitriol might be important in bolstering of resistance to tuberculosis[25]. Therefore, we applied three doses of calcitriol soaking (25/250/2500 µg/L per day) to investigate its prophylactic effect against *M. marinum* challenge. In accordance with previous reports[26, 27], low-dose and high-dose calcitriol soaking exerted anti-mycobacterium and anti-inflammatory effects. However, moderate-dose prophylactic soaking (250 µg/L per day) unexpectedly had no effect on the bacterial load and even exerted pro-inflammatory effects in *M. marinum* challenged zebrafish (Fig. 1d-f). This complicated dose-dependent anti-mycobacterium and anti-inflammatory effects of calcitriol may be one of the reasons for the puzzle in the benefit of Vitamin D in terms of tuberculosis treatment outcomes.

We next performed metabolomic analysis to investigate the underlying mechanism of the complicated dose-dependent anti-mycobacterium and anti-inflammatory effects of prophylactic calcitriol soaking. A total of 29 metabolites with between-group altered abundances were identified, including 41.8% (21/29) amino acids or the derivatives (L-Histidine, L-Threonine, Tryptamine, Taurine, L-Allotheta, Guanidoacetic acid, Putrescine, Creatine, N-Acetyl-L-alanine, Betaine, Dimethylglycine, and Propionylglycine), 10.3% (3/29) choline and its derivatives, 6.9% (2/29) the derivatives of fatty acids (2-Hydroxybutyric acid, and p-Hydroxyphenylacetic acid), 3.4% (1/29)the derivatives (D-Glucuronic acid), and 20.7% (6/29) other metabolites (N-Acetylgalactosamine, N-Acetylneuraminic acid, Mannitol, Deoxyuridine, Carnosine and Methylguanidine). In accordance with our findings, Vitamin D combined with one amino acid, L-arginine, is reported to be a potential adjunctive immunotherapy in tuberculosis[28], and the administered dose mattered[29]. Purine metabolism has been correlated with cytokine production in patients with fibro-cavernous pulmonary tuberculosis[30]. Fatty acid metabolism was reported to be influenced by Vitamin D deficiency and consequently increased the risk of tuberculosis infection[31]. N-Acetylgalactosamine, a substrate of arylsulfatase B (N-acetylgalactosamine-4-sulfatase), was proved to contribute to intracellular oxygen signaling and influence hypoxia[32], mannitol, an inhibitor of CYP2E1[33], could improve lung function in cystic fibrosis[34], Deoxyuridine troposphere nucleotidohydrolase, using deoxyuridine as a substrate, is a drug target against mycobacterium infections[35], Carnosine is an endogenous antioxidant widely distributed in excitable tissues[36], owning cytoprotective properties in primary cultured rat hepatocytes[37], Methylguanidine could inhibit lymphocyte transformation in vitro[38]. The concordance between the findings in previous reports and the present study suggested that the *M. marinum* challenged zebrafish is a suitable model in mimicking tuberculosis infection in human.

The glycine, serine, and threonine metabolism pathway (Fig. 4b-c) was predicted by the 22 metabolites co-varied with the doses of calcitriol and the 7 metabolites co-varied with the bacterial load and the
inflammatory factors (Fig. 3), respectively. According to the variation trends of the two metabolite groups, we speculated that the glycine, serine, and threonine metabolism pathway was regulated by prophylactic calcitriol soaking and was in response to the dose-dependent anti-mycobacterium and anti-inflammatory effects. In agreement with our finding, knockout of Vitamin D receptor resulted in upregulated glycine, serine, and threonine metabolism in murine intestinal microbiome[39]. Inhibiting of Vitamin D through massive small bowel resection could also increase the expression of glycine, serine, and threonine metabolism[40]. Glycine, serine and threonine metabolism could potentiate kanamycin-mediated killing of *Edwardsiella piscicida*[41], and confound efficacy of complement-mediated killing of difficult-to-treat pathogenic strains of bacteria[42]. The finding of the present study and previous reports suggested that glycine, serine, and threonine metabolism potentially contribute to the anti-mycobacterium and anti-inflammatory effects of calcitriol.

As a shortcoming of the present study, how the glycine, serine, and threonine metabolism pathway was altered by calcitriol soaking and responded to *M. marinum* challenge was not determined, which was essential for understanding the dose-dependent anti-mycobacterium effect of prophylactic calcitriol. Further studies are recommended to reveal the underlying pathways of calcitriol against mycobacterium infection through glycine, serine, and threonine metabolism.

In summary, the present study observed dose-dependent anti-mycobacterium and anti-inflammatory effects of prophylactic calcitriol soaking in *M. marinum* challenged adult zebrafish, with moderate-dose calcitriol exerted opposite effects to low- dose and high-dose calcitriol in bacterial load abundances of and inflammatory factors. Further metabolomic analysis indicated that the glycine, serine, and threonine metabolism pathway was induced by prophylactic calcitriol soaking and was in response to the dose-dependent anti-mycobacterium and anti-inflammatory effects of calcitriol.

**Declarations**

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**Author contributions**

Jianguo Li, Conceptualization, writing-original draft. Zhen Li, Investigation and data curation. Zefeng Gao, Investigation. Juan Xia, Investigation. Jialei Zhang, Investigation. Jia Cui, Investigation. Changxin Wu, Writing- review & editing, supervision.

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Availability of data and materials

The dataset supporting the conclusions of this article is included within the article.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki. The experimental procedures were approved by the ethics committee at Shanxi University.

Consent for publication

Not applicable.

Competing interests

No potential conflicts of interest were disclosed by authors.

References

1. Norman AW: 
   1,25-dihydroxyvitamin D3: a kidney-produced steroid hormone essential to calcium homeostasis. 
   *Am J Med* 1974, **57**:21-27.

2. Shiizaki K, Hatamura I, Imazeki I, Moriguchi Y, Sakaguchi T, Saji F, Nakazawa E, Kato S, Akizawa T, Kusano E: 
   Improvement of impaired calcium and skeletal homeostasis in vitamin D receptor knockout mice by a high dose of calcitriol and maxacalcitol. 
   *Bone* 2009, **45**:964-971.

3. Junaid K, Rehman A, Jolliffe DA, Saeed T, Wood K, Martineau AR: 
   Vitamin D deficiency associates with susceptibility to tuberculosis in Pakistan, but polymorphisms in VDR, DBP and CYP2R1 do not. 
   *BMC Pulm Med* 2016, **16**:73.

4. Huang SJ, Wang XH, Liu ZD, Cao WL, Han Y, Ma AG, Xu SF: 
   Vitamin D deficiency and the risk of tuberculosis: a meta-analysis. 
   *Drug Des Devel Ther* 2017, **11**:91-102.

5. Chan TY: 
   Vitamin D deficiency and susceptibility to tuberculosis. 
   *Calcif Tissue Int* 2000, **66**:476-478.

6. Gurjav U, Ankhbat M, Ganbaatar G, Batjarga K, Ochirbat B, Baigal D, Jargalsaikhan B, Munkhjargal O, Bolormaa S, Yansanjav N, et al: 
   Vitamin D deficiency is associated with tuberculosis infection among household contacts in Ulaanbaatar, Mongolia. 
   *Int J Tuberc Lung Dis* 2019, **23**:919-923.

7. McArdle AJ, Keane D, Seddon JA, Bernatoniene J, Paton J, McMaster P, Williams A, Williams B, Kampmann B: 
   Vitamin D deficiency is associated with tuberculosis disease in British children. 
   *Int J Tuberc Lung Dis* 2020, **24**:782-788.

8. Bhaumik S: 
   Vitamin D supplementation for tuberculosis. 
   *Indian Pediatr* 2012, **49**:931.

9. Binet L, Bour H: 
   Trials for the treatment of pulmonary tuberculosis with nebulization of vitamin D2. 
   *Prog Med (Paris)* 1946, **74**:579-581.

10. Wejse C, Gomes VF, Rabna P, Gustafson P, Aaby P, Lisse IM, Andersen PL, Glerup H, Sodemann M: 
    Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-
11. Martineau AR, Timms PM, Bothamley GH, Hanifa Y, Islam K, Claxton AP, Packe GE, Moore-Gillon JC, Darmalingam M, Davidson RN, et al: **High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial.** *Lancet* 2011, 377:242-250.

12. Tukvadze N, Sanikidze E, Kipiani M, Hebar G, Easley KA, Shenvi N, Kempker RR, Frediani JK, Mirtskhulava V, Alvarez JA, et al: **High-dose vitamin D3 in adults with pulmonary tuberculosis: a double-blind randomized controlled trial.** *Am J Clin Nutr* 2015, 102:1059-1069.

13. Ganmaa D, Munkhzul B, Fawzi W, Spiegelman D, Willett WC, Bayasgalan P, Baasansuren E, Buyankhishig B, Oyun-Erdene S, Jolliffe DA, et al: **High-Dose Vitamin D3 during Tuberculosis Treatment in Mongolia. A Randomized Controlled Trial.** *Am J Respir Crit Care Med* 2017, 196:628-637.

14. Bekele A, Gebreselassie N, Ashena A, Kassa E, Aseffa G, Amogne W, Getachew M, Aseffa A, Worku A, Raqib R, et al: **Daily adjunctive therapy with vitamin D3 and phenylbutyrate supports clinical recovery from pulmonary tuberculosis: a randomized controlled trial in Ethiopia.** *J Intern Med* 2018, 284:292-306.

15. Ganmaa D, Uyanga B, Zhou X, Gantsetseg G, Delgerekh B, Enkhmaa D, Khulan D, Ariunzaya S, Sumiya E, Bolortuya B, et al: **Vitamin D Supplements for Prevention of Tuberculosis Infection and Disease.** *N Engl J Med* 2020, 383:359-368.

16. Reuter A, Furin J: **The problem with vitamin D supplementation for tuberculosis.** *Lancet HIV* 2020, 7:e450-e451.

17. Cakici C, Yigitbasi T, Ayla S, Karimkhani H, Bayramoglu F, Yigit P, Kilic E, Emekli N: **Dose-dependent effects of vitamin 1,25(OH)2D3 on oxidative stress and apoptosis.** *J Basic Clin Physiol Pharmacol* 2018, 29:271-279.

18. Rodriguez-Lecompte JC, Yitbarek A, Cuperus T, Echeverry H, van Dijk A: **The immunomodulatory effect of vitamin D in chickens is dose-dependent and influenced by calcium and phosphorus levels.** *Poult Sci* 2016, 95:2547-2556.

19. Burt LA, Billington EO, Rose MS, Raymond DA, Hanley DA, Boyd SK: **Effect of High-Dose Vitamin D Supplementation on Volumetric Bone Density and Bone Strength: A Randomized Clinical Trial.** *JAMA* 2019, 322:736-745.

20. Ryan KJ, Daniel ZC, Craggs LJ, Parr T, Brameld JM: **Dose-dependent effects of vitamin D on transdifferentiation of skeletal muscle cells to adipose cells.** *J Endocrinol* 2013, 217:45-58.

21. Liang S, Hou Z, Li X, Wang J, Cai L, Zhang R, Li J: **The fecal metabolome is associated with gestational diabetes mellitus.** *RSC Advances* 2019, 9.

22. Amin Z, Rumende CM: **The effect of vitamin D as adjuvant therapy in pulmonary tuberculosis with moderate-advance lesion.** *Acta Med Indones* 2006, 38:1-2.

23. Periyasamy KM, Ranganathan UD, Tripathy SP, Bethunaickan R: **Vitamin D - A host directed autophagy mediated therapy for tuberculosis.** *Mol Immunol* 2020, 127:238-244.
24. Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, Ochoa MT, Schauber J, Wu K, Meinken C, et al: Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 2006, 311:1770-1773.

25. Aibana O, Huang CC, Aboud S, Amedo-Pena A, Becerra MC, Bellido-Blasco JB, Bhosale R, Calderon R, Chiang S, Contreras C, et al: Vitamin D status and risk of incident tuberculosis disease: A nested case-control study, systematic review, and individual-participant data meta-analysis. *PLoS Med* 2019, 16:e1002907.

26. Greenstein RJ, Su L, Shahidi A, Brown WD, Clifford A, Brown ST: Unanticipated Mycobacterium tuberculosis complex culture inhibition by immune modulators, immune suppressants, a growth enhancer, and vitamins A and D: clinical implications. *Int J Infect Dis* 2014, 26:37-43.

27. Khoo AL, Chai LY, Koenen HJ, Oosting M, Steinmeyer A, Zuegel U, Joosten I, Netea MG, van der Ven AJ: Vitamin D(3) down-regulates proinflammatory cytokine response to Mycobacterium tuberculosis through pattern recognition receptors while inducing protective cathelicidin production. *Cytokine* 2011, 55:294-300.

28. Ralph AP, Kelly PM, Anstey NM: L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis. *Trends Microbiol* 2008, 16:336-344.

29. Ralph AP, Waramori G, Pontororing GJ, Kenangalem E, Wiguna A, Tjitra E, Sandjaja, Lolong DB, Yeo TW, Chatfield MD, et al: L-arginine and vitamin D adjunctive therapies in pulmonary tuberculosis: a randomised, double-blind, placebo-controlled trial. *PLoS One* 2013, 8:e70032.

30. Dyakova M, Zhuravlev V, Esmedlyaeva D, Perova T: Role of Purine Metabolism Enzymes in Immune Pathogenesis of Fibro-Cavernous Pulmonary Tuberculosis. *Med Immunol (Russia)* 2016, 18:85-90.

31. Wang Q, Ma A, Schouten EG, Kok FJ: A double burden of tuberculosis and diabetes mellitus and the possible role of vitamin D deficiency. *Clin Nutr* 2020.

32. Bhattacharyya S, Tobacman JK: Hypoxia reduces arylsulfatase B activity and silencing arylsulfatase B replicates and mediates the effects of hypoxia. *PLoS One* 2012, 7:e33250.

33. Shih TY, Ho SC, Hsiong CH, Huang TY, Hu OY: Selected pharmaceutical excipient prevent isoniazid and rifampicin induced hepatotoxicity. *Curr Drug Metab* 2013, 14:720-728.

34. Jaques A, Daviskas E, Turton JA, McKay K, Cooper P, Stirling RG, Robertson CF, Bye PTP, LeSouef PN, Shadbolt B, et al: Inhaled mannitol improves lung function in cystic fibrosis. *Chest* 2008, 133:1388-1396.

35. Singh V, Brecik M, Mukherjee R, Evans JC, Svetlikova Z, Blasko J, Surade S, Blackburn J, Warner DF, Mikusova K, Mizrahi V: The complex mechanism of antimycobacterial action of 5-fluorouracil. *Chem Biol* 2015, 22:63-75.

36. Caruso G, Fresta CG, Musso N, Giambirone M, Grasso M, Spampinato SF, Merlo S, Drago F, Lazzarino G, Sortino MA, et al: Carnosine Prevents Abeta-Induced Oxidative Stress and Inflammation in Microglial Cells: A Key Role of TGF-beta1. *Cells* 2019, 8.

37. Eftekhari A, Heidari R, Ahmadian E, Eghbal M: Cytoprotective Properties of Carnosine against Isoniazid-Induced Toxicity in Primary Cultured Rat Hepatocytes. *Pharm Sci* 2018, 24:257-263.
38. Traeger J, Jouraine J, Revillard J, Brochier J, Natarro J: Role of methylguanidine and middle molecules in the immunodeficiency secondary to uremia. Proc 6th Int Congr Nephrol Florence S Karger, Basel 1976:584.

39. Jin D, Wu S, Zhang YG, Lu R, Xia Y, Dong H, Sun J: Lack of Vitamin D Receptor Causes Dysbiosis and Changes the Functions of the Murine Intestinal Microbiome. Clin Ther 2015, 37:996-1009 e1007.

40. Hadjittof C, Coran AG, Mogilner JG, Pollak Y, Matter I, Sukhotnik I: Dietary supplementation with vitamin D stimulates intestinal epithelial cell turnover after massive small bowel resection in rats. Pediatr Surg Int 2013, 29:41-50.

41. Ye JZ, Lin XM, Cheng ZX, Su YB, Li WX, Ali FM, Zheng J, Peng B: Identification and efficacy of glycine, serine and threonine metabolism in potentiating kanamycin-mediated killing of Edwardsiella piscicida. J Proteomics 2018, 183:34-44.

42. Cheng ZX, Guo C, Chen ZG, Yang TC, Zhang JY, Wang J, Zhu JX, Li D, Zhang TT, Li H, et al: Glycine, serine and threonine metabolism confounds efficacy of complement-mediated killing. Nat Commun 2019, 10:3325.

Figures
Figure 1

Experimental design diagram and phenotypic variations in M. marinum challenged adult zebrafish receiving prophylactic calcitriol soaking. (a) The experimental design of the present study was as follows: 30 tails zebrafish were randomly divided into five groups (6 tails per group); four groups received calcitriol soaking from 30 dpf (day post fertilization) to 60 dpf with doses of 0, 25, 250, 2500 μg/L per day, respectively. The rest one group zebrafish was taken as control. At the time point of 60 dpf, the four
zebrafish groups received calcitriol soaking were challenged with M. marinum (100 CFU/fish) through tail vein injection, and monitored for 15 days. At the time point of 75 dpf, all of the five groups of zebrafish were suicided and stored at -80 °C for further investigation. Body weight (b), bacterial load (c), relative abundances of IL-1β (d), IFN-γ (e) and TNF-α (f) in samples collected at 75 dpf were measured and expressed with Violin plot. The violin plot outlines illustrate kernel probability density, with the top and bottom edges define the 95% confidence interval. The dashed lines inside a plot indicate 75% quartile, median, and 25% quartile of the corresponding data from top to bottom. Between-group statistical significances were evaluated by unpaired t-test with Welch's correction (for body weight and bacterial load) or Mann-Whitney test (for the relative abundances of inflammatory factors). * P < 0.05, ** P < 0.01, *** P< 0.001.
Figure 2

Metabolomic analysis of the *M. marinum* challenged adult zebrafish receiving prophylactic calcitriol soaking. (a) PCA score plot for the 1H-NMR metabolomic profiling of *M. marinum* challenged adult zebrafish receiving different dose of prophylactic calcitriol soaking. Low-dose: 25 μg/L/day, moderate-dose: 250 μg/L/day, high-dose: 2500 μg/L/day. The first two principal components explained 21% and 14.3% of the total variances, respectively. (b) OPLS-DA score plot for the metabolome of *M. marinum*.
challenged adult zebrafish receiving different dose of prophylactic calcitriol soaking. The horizontal axis represents the predicted score of the first component, which explained 66.7% of the between-group variations (R2X=0.667). The vertical axis represents the orthogonal principal component score, which explained 83.3% of the within-group variations (R2Y=0.833). The OPLS-DA model explained 77% of the total variations (Q2=0.770). Pairwise OPLS-DA and the derived S-Plot of low-dose vs moderate-dose calcitriol (c, d), moderate-dose vs high-dose calcitriol (e, f), and low-dose vs high-dose (g, h). The S-plot shows covariance coefficient (p) and correlation coefficient (p(corr)) of the features from 1H-NMR metabolomic profiling. Each point in the S-plot represents a 1H-NMR feature and is colored according to the value of correlation coefficient. Cut-off values for p > |0.1| and p(corr) > |0.532| were used.
Figure 3

Relative abundances of the altered metabolites among M. marinum challenged zebrafish receiving different doses of calcitriol soaking. The altered metabolites were screened by pairwise comparison among different groups (low-/moderate-/high-dose of calcitriol soaking) of M. marinum challenged zebrafish. Metabolites with relative abundance ranging from 0.001 to 0.003 (a), from 0.002 to 0.006 (b), from 0.01 to 0.04 (c), from 0.005 to 0.015 (d), from 0.01 to 0.08 were displayed separately. Metabolite name following an asterisk indicates that the metabolite was co-varied with the bacterial load and inflammatory factors. Between-group statistical significances were evaluated by Mann-Whitney test. *P < 0.05, **P < 0.01, ***P < 0.001.
Glycine, serine and threonine metabolism was inferred from the metabolomic shift induced by prophylactic calcitriol soaking of M. marinum challenged zebrash. (a) Heatmap of the spearman rank correlations among the altered metabolites, the body weight, the bacterial load and the inflammatory factors in M. marinum challenged zebrash receiving different doses of prophylactic calcitriol soaking. The size of a square represents the range of the corresponding correlation coefficient (p). The color of a square represents the range of the corresponding p in according to the color bar to the right of the matrix. * represents the result of statistical test: * P < 0.05, ** P < 0.01. (b) Pathway prediction based on the altered metabolites co-varied with the doses of calcitriol in M. marinum challenged zebrash. Only pathways with P values lowers than 0.05 in hypergeometric test for over representation analysis were labelled. The detailed information for all pathways could be observed in Table S3. (c) Pathway prediction based on the altered metabolites co-varied with the bacterial load and inflammatory factors in M. marinum challenged zebrash. Only pathways with P values lowers than 0.05 in hypergeometric test for over representation analysis were labelled. The detailed information for all pathways could be observed in Table S4. (d) Glycine, serine and threonine metabolism was co-predicted by both the metabolite group co-varied with the doses of calcitriol and the metabolite group co-varied with the bacterial load and inflammatory factors in M. marinum challenged zebrash. The metabolites from the former group in the pathway were labelled in pink, the metabolites from the later group were labelled in blue.

Supplementary Files

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