Cell. Mol. Biol. 58 (Supp): OL1688-OL1694
Published on June 15, 2012
DOI 10.1170/196

Cellular & Molecular Biology
http://www.cellmolbiol.com

EFFECT OF INTERACTION OF VITAMIN C ON MACROPHAGE IMMUNE RESPONSE TO INFECTION WITH *Mycobacterium bovis*

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Abstract

Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis* affecting humans and livestock. Like *Mycobacterium tuberculosis* (M.tb), *M. bovis* can persist in cattle without causing overt symptoms after entering a non-replicating persistent (NRP) state. Given that M.tb enters NRP under stress conditions, we sought to find the effects of vitamin C (VC) on *M. bovis in vitro* and *in vivo* (VC could mimic stresses like hypoxia by O_2 scavenging and acidic conditions in phagosome). *M. bovis* was cultured in a medium with VC for 48h. The differential expression of five genes (*dosR*, *dosS, dosT*, *icl*, and *hspX* of *M. bovis*) implicated in the *M. bovis* NRP state was measured with real-time quantitative PCR. Expression of all five genes was increased by VC. Relative to the control, VC-exposed bacteria appeared smaller and more rounded in shape with a much thicker inner envelope. A lower number of viable bacteria were found in comparison with those of the control. We infected macrophage cell line ANA-1 with *M. bovis* and cultured it in VC-added medium (MC group) for 24h and 48h. Expression of *il-10, il-6, tnf-α,* and *il-β* was examined and compared with expression by cells infected by *M. bovis* only without VC treatment (MB group), uninfected cells in the medium treated with VC (VC group), and cells in the medium only without VC. *Il-1β*, *tnf-α*, and *il-6* transcription were up-regulated significantly in MC group. IL-10 gene expression in MB and MC groups was less than in the control at 24h, but that of MC group increased more than the MB group at 48h. The numbers of intracellular *M. bovis* in the MC group were lower than that in the other groups. Slower growth was found in VC-treated *M. bovis*, and macrophages were more bactericidal for intracellular VC-stimulated *M. bovis* than for *M. bovis* with no VC treatment.

Key words: *Mycobacterium bovis*, Non-replicating state, macrophages, cytokines, vitamin C.

Article information

Received on April 5, 2012
Accepted on May 31, 2012

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INTRODUCTION

A relatively small proportion of human patients carrying *Mycobacterium tuberculosis* progress to full disease for the bacteria surviving in the host for decades in a non-replicating persistent (NRP) state (31). Little is known about the features that allow bacteria to remain within the host in this way. *M. bovis* is the causative agent of tuberculosis in cattle which remains an important zoonotic infection in addition to being a significant world-wide economic agricultural problem. About 1%–2% of human TB cases in developed countries are caused by *M. bovis* (18). In addition to studying the basis of latency in *M. bovis* in its own right such studies could additionally shed light on latency in M.tb.

To elucidate the basis of the NRP mechanism, *in vitro* conditions are studied to mimic the situation *in vivo*. M.tb is induced to enter the NRP state under low pH or oxygen stress conditions (30, 4). These studies generally show that dormant-state M.tb is induced by the *dosR* regulon. Expression of the *dosR* regulon of M.tb or *M. bovis* is initiated during transition into dormancy, and is regulated by the response regulator *dosR* (*devR*, *Rv3133c*), which is activated by two sensor histidine kinases, *dosT* (Rv2027c) and *dosS,* (DevS, Rv3132c) (20, 22, 7).

L-ascorbic acid (Vitamin C or VC) has a special role in redox interrelationships as an antioxidant and enzyme co-factor (16). It is reported that its deficiency could result in immune defect and it also could enhance the proliferative T lymphocytes in vitro as well as the tuberculin skin hypersensitivity in vivo (13). Some TB patients are characterized by a lack of sufficient VC (1). What's more, Macrophage is of importance in oxidative stress developing for antimicrobial and antitumoural defence within the cell-mediated immune response, while simulated macrophages play a crucial role in cardiovascular disease (23). Taneja et al. found that VC induced dormancy in M.tb, this process coinciding with up-regulation of expression in *dosR, dosS* but not *dosT* (27).

Given the similarities between M.tb and *M. bovis,* we hypothesized that VC might induce a dormant-like state of *M. bovis*. The cytokine production of the macrophage will be different when they are treated with VC post infection of *M. bovis*. First, we examined changes in the expression of selected genes related to phenotypic characteristics of VC-treated *M. bovis*. Results indicated some genes were induced to different degrees, including *dosR*, *dosT*, and *dosS*. What's more, we found a significant difference in gene expression of cytokines related to the macrophage immune response and the number of intracellular bacteria after treatment with VC post infection, which was a new discovery in the effects of VC on *M. bovis* infection. This

study could clearly lead to a better understanding of the nature of the interaction between *M. bovis* and the macrophage and possibly suggest approaches to clinical intervention.

MATERIALS AND METHODS

Bacterial strains and treatment with VC in vitro

All experiments were carried out using the *M. bovis* Beijing strain (strain 93006) from the China Institute of Veterinary Drug Control (CVCC, Beijing, China). *M. bovis* was cultured in Middlebrook 7H9 broth (Difco) supplemented with 10% (v/v) ADC (0.5g/l BSA, 0.2g/l dextrose and 0.085g/l NaCl) and 0.05% (v/v) Tween80, incubated at 37℃ with shaking for 4-6 weeks. All contents of the medium are free of VC unless adding it according to the protocol. Viable bacterial numbers were counted by plating 100uL of serial dilutions onto Middlebrook 7H11 agar containing 10% (v/v) ADC plus 0.05% (v/v) Tween80. Broth cultures were aliquoted into six 3mL-portions in 15mL tubes. L-ascorbic acid was dissolved into ultrapure water under axenic conditions for a final concentration of 1M. Cultures were treated with VC immediately (to prevent possible oxidation) at 10 mM final concentration for 8h under the same cultural conditions (27), and then compared with untreated cultures. Comparisons were carried out at least three times.

M. bovis-infected ANA-1 and RNA isolation

ANA-1 is a murine, macrophage-like cell line. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, and plated in 12-well culture plates, 1×10^6 cells per well, for 12h at 37 C, 5% CO_2. All contents of the medium are free of VC unless adding it according to the protocol. Infections were carried out for 3h with *M. bovis* at a 10:1 multiplicity of infection. Wells were subsequently washed with incomplete RPMI 1640 medium and VC was then added to the test wells (post-infection or control) at a final concentration of 2 mM. The four experimental groups included: *M. bovis*-infected cells (MB group), cells infected by *M. bovis* for 3h and then treated with VC (MC group), cells cultured in medium added with VC (VC group), and cells cultured in medium only (control group). Total RNA of each was isolated using the RNApure High-purity Total RNA Rapid Extraction Kit (Aid Lab, China) at 24h and 48h, and stored in RNase-free water at -80℃.

RNA extraction from M. bovis and cDNA preparation

RNA was isolated from *M. bovis* using the following steps. Bacterial cells of each sample (about 3×10^8CFU/ml) were harvested by centrifugation at 2,000g for 10min followed by addition of 0.5ml silica beads (0.1mm diameter) (Biospec). Trizol (Invitrogen, USA) was added to the beads at a ratio of 1:1, and mixed thoroughly. Cells were then disrupted for 3min using a Mini Bead-beater cell disruptor (Shimadzu, Japan) with 0.1mm glass beads. Total RNA was purified using the RNApure High-purity Total RNA Rapid Extraction Kit (Aid Lab, China) and stored in RNase-free water at -80℃. *M. bovis* or ANA-1 cell RNA (500ng-1000ng) was reverse transcribed to cDNA using the RevertAid first-strand cDNA synthesis kit (Fermentas, Lithuania).

Real-time quantitative PCR

For each group, expression levels of the selected genes (*dosR*, *dosS*, *dosT*, *icl*, and *hspX* of *M. bovis*; IL-10, IL-6, TNF-α, and IL-1β of ANA-1 cell) were examined using real-time quantitative PCR. Expression levels were detected using the DNA Engine Opticon TM2 fluorescence detection system (MJ Research Inc, USA) and SYBR Green (Tiangen, China). Transcript levels between various RNA samples were normalized using 16S rRNA or b-actin. The specific gene primer pairs designed according to Genebank-recorded sequences are shown in Table 1. Real-time quantitative PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method, and differences between the samples were analyzed using a t-test with SPSS software (Spssstatistics 17.0).

Acid-fast staining

Phenotypical differences (i.e. cell shape, bacteria size) between the two groups (VC-treated group and control) were observed using acid-fast staining and microscopy. Cells from each group were washed twice with PBS to remove culture medium traces. Bacteria were then stained using the acid-fast staining method according to a standard Ziehl–Neelsen stain protocol.

Viable bacterial counts of M. bovis in vitro

For bacteria counting of VC-treated *M. bovis in vitro* at 0h, 24h, and 48h after adding VC, samples were serially diluted in PBS and plated onto Middlebrook 7H11 plates containing 10% (v/v) ADC and 0.05% (v/v) Tween80 for bacterial enumeration. Colony forming units (CFU) were counted after incubation at 37℃ for 3–4 weeks.

To count intracellular bacteria, ANA-1 cells cultured in 12-well plates were harvested by centrifugation at 500g for 3min at 24h and 48h for the four groups. Cells from each well were washed three times with PBS to remove extracellular bacteria and then lysed with a 0.1% Triton X-100 solution, serially diluted in PBS, and plated onto Middlebrook 7H11 plates (29). Cells were then cultured and CFUs counted as described above.

Transmission electron microscopy

Briefly, bacterial cells of the two groups (VC-treated group and control) were harvested at 2,000g for 10min and washed twice with PBS, followed by centrifugation before fixation in 4% glutaraldehyde buffer for 2d at room temperature. They were then processed for electron microscopy as previously described (19). Electron microscopy sections were prepared and observed by transmission electron microscopy.

RESULTS

Gene expression changes induced by ascorbic acid

To estimate *dosR*, *dosS*, *dosT*, *icl*, and *hspX* gene expression changes, total RNA was extracted from VC-treated cultures of *M. bovis* and compared with untreated cultures using real-time quantitative PCR analysis.

The results are shown in Figure 1. The broken line shows the boundary between up-regulated genes (above the line) and down-regulated genes (below the line). The results showed that *dosR*, *dosS*, and *dosT* gene expressions were induced 1.9 fold, 2.3 fold, 18.89 fold respectively by VC. These genes were associated with *M. bovis* dormancy. The

Table 1. Real-time quantitative PCR primer pairs and amplicon size for all analyzed genes.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Amplicon size (bp)
dosR	tcttcaccgtcttttcggct	gcggatatgtcgtcaaagac	252
dosS	tcgatgcacaagtcgtcgtc	gcaccagcgttcaattcgtg	160
dosT	acccgcacatcatcctcg	gaatccatctacagcagcatcg	308
hspX	accgtgcgaacgaaggaac	tgcggctggaagacgagat	187
icl	agcaggaatgggacacgaac	gccagcccgacaggtagat	240
16s rRNA	acgtgggtactaggtgtgggtttc	tctgcgattagcgactaagacttca	543
il-1β	gcctcaaaggaaagaatcta	aaacagtccagcccatactt	297
tnf-α	ggcggtgcctatgtctca	cacttggtggtttgctacg	221
il-6	tgccttcttgggactgat	ctggctttgtctttcttgtt	384
il-10	cagccaggtgaagactttct	atggccttgtagacaccttg	330
b-actin	tgctgtccctgtatgcctctg	ttgatgtaccgcacgatttcc	223

levels of *icl* (10.9 fold) and *hspX* (6.7 fold) were higher than those of the control.

To detect the effect of VC on macrophage bactericidal ability, ANA-1 cells were infected with *M. bovis* for 3h. After washing off the extracellular bacteria, VC was added to the medium and kept for 24h and 48h. IL-10, IL-6, TNF-α, and IL-1β expressions of ANA-1 cells were examined using real-time quantitative PCR. Results (Figure 4) showed that pro-inflammatory cytokines, IL-6, TNF-α, and IL-1β, were more activated in MC than MB groups at 24h or 48h. Gene expression of IL-1β was significantly ($p<0.05$) up-regulated more in MC than in other groups at 24h and 48h. IL-6 was induced more in both MC and MB groups at 24h, and the level in MC was higher than in the MB group. At 48h, the IL-6 gene expression level decreased sharply ($p<0.05$) and arrived at the level same as control group. IL-6 level increased constantly in VC group within 48h. The expression of TNF-α changed differently. At 24h, TNF-α expression in the MB group was down-regulated compared to control, while TNF-α in the MC group was up-regulated more than in control. However, TNF-α in MB group increased while TNF-α in the MC group decreased, both arriving at a similar level at 48h. At 24h, IL-10 gene expression in the MB and MC groups was lower than in the control group, expression in both groups increased significantly ($p<0.05$) at 48h. The IL-10 expression level in the MC group was higher than other groups at 48h. Further, VC promoted cell production of pro-inflammatory cytokines like IL-6, TNF-α, and IL-1β, to different degrees. However, IL-10 gene expression was lower in VC group than in the control at 24h and 48h.

Bacterial enumeration in vitro

To analyze changes in bacterial growth characteristics at 0h, 24h, and 48h post-VC-treatment *in vitro*, *M. bovis* CFU were enumerated after incubation at 37℃ for 3~4 weeks. When bacteria were exposed to VC, the initial CFU was Log_{10} 5.75±0.04 at 0h, decreased continually to Log_{10} 5.29±0.15 at 24h, and to 5.01±0.03 at 48h. *M. bovis* grew quickly in medium without-VC and the viable count was Log_{10} 6.62±0.03 logs at 24h, increasing to 7.94±0.01 at 48h (Figure 2A). The differences between VC-treated and control groups were statistically significant at 24h and at 48h.

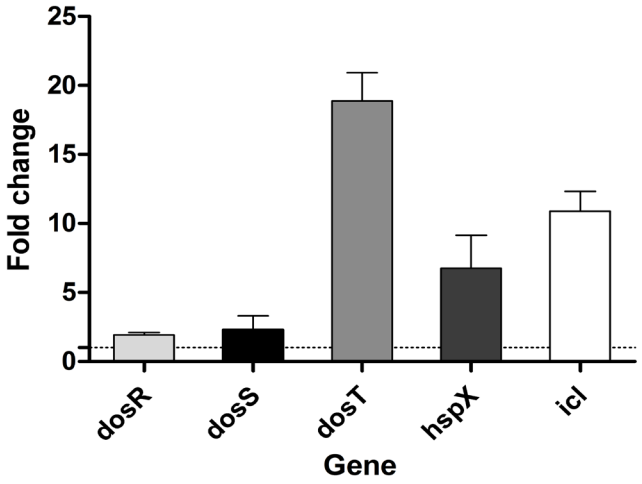

Figure 1. Gene expression changes measured by real-time quantitative PCR in mRNA levels of selected genes after VC-exposure. RT-PCR analysis of selected genes was carried out with total RNA isolated from VC-treated *M. bovis* cultures *in vitro* for 8 hours with control cultures remaining untreated. Every bar shows the fold induction of a particular gene with respect to untreated cultures. The fold induction of each gene expression level is a ratio of 16s rRNA-normalized transcription in VC-treated or untreated cultures. The results are shown as mean±SD of 3 independent samples each analyzed in 3 replicate assays.

Acid-fast staining for bacterium morphology

Morphological differences between groups were observed using acid-fast staining. After VC-treatment for 48h, bacterial cells appeared smaller and more rounded in shape than the control cells (Figure 3A, B). Images are representative of the bacilli in at least 10 different microscopic fields.

Transmission electron microscopy

VC-treated bacteria were smaller than control bacteria (Figure 3C, D). Control bacteria also contained more vesicles than did treated bacterial cells. Cell walls of the treated group were much thicker than those of the control group.

Enumeration of intracellular M. bovis

Growth and survival of intracellular *M. bovis* were assessed by counting ANA-1 intracellular bacteria (Figure

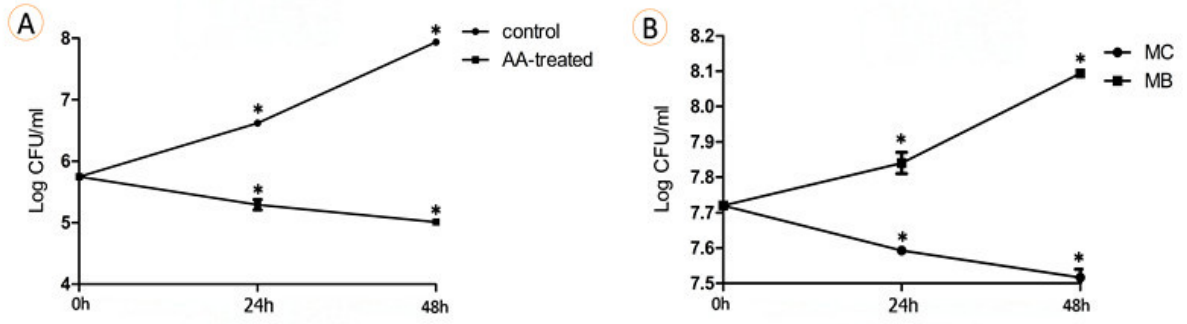

Figure 2. A: Bacteria growth arrest caused by VC in *M. bovis* cultures. The CFU numbers were determined at 0h, 24h, and 48h after VC treatment. It shows the mean ±SD of 3 replicates of plates in each group for every experiment ('*', P-value<0.05). B: Growth arrest of intracellular bacteria caused by VC performed by CFU assessment. The intracellular *M. bovis* number count was determined at 0h, 24h, and 48h after stimulation with VC or not. It shows the mean±SD of 3 replicates of plates in each group for every experiment ('*', P-value<0.05).

2B). Intracellular viable counts were Log_{10} 7.59±0.01 at 24h, which decreased significantly to 7.52±0.04 at 48h in the MC group. The changes in CFU in the MB group were different, namely Log_{10} 7.84±0.05 at 24h, which increased significantly to 8.09±0.02 at 48h. No bacteria were cultured in the groups treated with VC and medium only. The results were consistent with poor growth resulting from culturing *M. bovis in vitro* with VC.

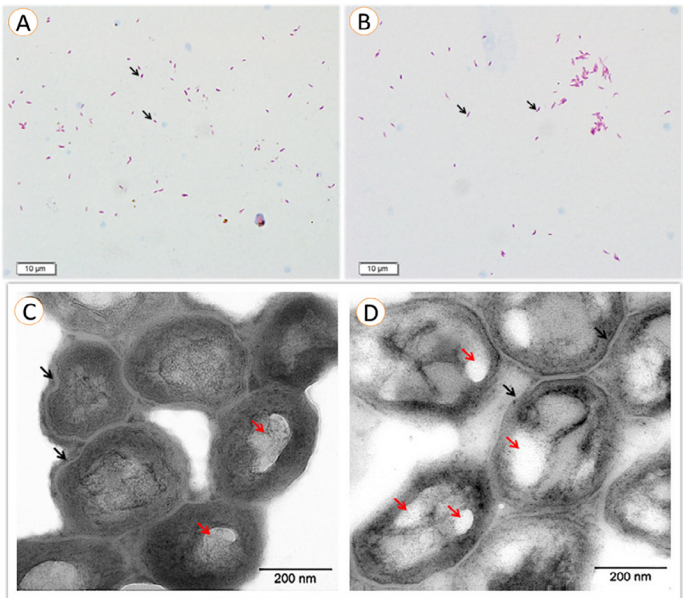

Figure 3. Bacteria morphology. *M. bovis* (red) cells were stained with Ziehl–Neelsen stain and are shown as arrows point (A is VC-treated group, B is untreated). Magnification x1000. (C)Electron micrograph of VC-treated *M. bovis* or (D) untreated. Red arrows point to the vesicles and black ones point to the cell walls. Magnification ×120,000 (Scale bar, 200nm).

DISCUSSION

Tuberculosis remains a major global public health problem. Bovine tuberculosis threatens livestock and has serious implications for human health, where dormancy and persistent infection is a key characteristic of the disease. A number of studies have attempted to understand the dormancy mechanism (11, 3).

M.tb dormancy could be induced by VC in several ways. First of all, VC could scavenge O_2 in a rapid way and develop hypoxia consequence. DosR response regulator in M.tb is a very important regulon during hypoxia. It is crucial in adaptation of hypoxia. Taneja et al have discovered that VC rapidly induces the DosR regulon under aerobic conditions (27). In this study, we examined dosR, dosT and dosS gene expression level and these genes expression were obviously induced after addition of VC, indicating that interactions between VC and *M. bovis* was similar with that of M.tb. The 16-kDa alpha crystalline (Acr), encoded by hspX gene (Rv2031c), is the best-known member of the dosR regulon (21). HspX expression is greatly increased when stimulated by "stressed" conditions like hypoxia (25). In this study, HspX gene expression increased consistently when *M. bovis* was exposed to VC, which confirmed the induction of the dormancy regulon. Because the addition of VC causes the acidification of culture medium to pH~5.5, we confirmed that isolitrate lyase (ICL), a gene is acid-inducible and is required for non-replicating persistence of M.tb in macrophages and mice(5, 17), is up-regulated 10.9 fold in this study. ICL encoded isocitrate lyase which is a key enzyme of the glyoxylate pathway. Therefore, VC-treated *M. bovis* may enter the early period of non-replication quickly with the bacterial metabolism adjusted to accommodate the stressed condition.

Conditions experienced by bacteria in latency are very different from conditions to which bacteria are exposed during activation. The host immune system is less able to kill bacteria in granulomas in latent tuberculosis infection; bacteria are surrounded by macrophages, neutrophil, and T-cells with the granuloma sometimes completely walled off (31). To detect whether VC-treated *M. bovis in vitro* presented growth arrest, bacteria CFU were carried out. VC-exposed *M. bovis* grew less than those cultured in normal medium. Thus, VC was able to induce growth arrest of *M. bovis in vitro*. It is studied that some stress conditions such as oxygen deficient could induce growth arrest of M.tb. VC aqueous solution shows acidic and could induce O_2 consumption, which could have potentiated the adaptation of *M. bovis* to a dormant-like state, similar to the Wayne model (33).

M.tb exposed to nutrient-deficient cultures changed morphology and became smaller and more rounded in shape (12). Bacteria gradually lose acid-fast staining; this may be

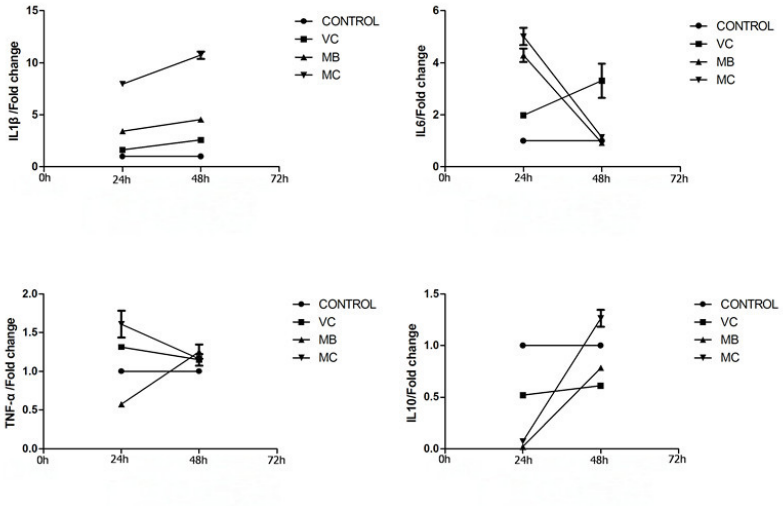

Figure 4. Gene expression changes measured by real-time quantitative PCR in mRNA levels of selected genes. RT-PCR analysis of selected genes was carried out with total RNA isolated from the four groups, MC, MB, VC and control (MC: treat with VC after stimulation with *M. bovis*, MB: stimulation with *M. bovis* only, VC: treated with VC only, control: medium only). Every point shows the fold induction of a particular gene with respect to untreated cultures. The fold induction of each gene expression level is a ratio of *b-actin*-normalized transcription in each group. The results are shown as mean±SD of 3 independent samples each analyzed in 3 replicate assays.

relevant to alterations occurring in the cell envelope structure (24). Here, as a kind of stress condition, we find that VC-treated *M. bovis* cells were smaller and more rounded in shape at 48h but did not completely lose the acid-fast property. They are bacteria were smaller in diameter and possessed a thicker cell envelope than non-treated bacteria, which was reported first time on the effect of VC on mycobacteria. Low et al indicated that mycobacteria could collect lipids from cytoplasm (i.e. triacylglycerol) when exposed to stress conditions (14). We thought that might be the main reason to cause cell wall to become thicker after VC treatment on *M. bovis*. Given the relative impenetrability of the mycobacterial envelope, this may explain the phenotype of growth arrest.

The effects of VC on immune functions were studied earlier in 1970's. From then on, many reports have involved in regulation of VC on immunity (28,15,32). VC is highly concentrated in leukocytes and is used rapidly during infection. It has been defined as a stimulant of leukocyte functions, especially of neutrophil and monocyte movement. Vitamin C supplementation did not increase PBMC production of IL-1, IL-6 and TNF-α, but when VC combined VE, the production of IL-1, IL-6 and TNF-αwere significantly promoted (10). In another study, VC inhibited the LPS-induced member of monocytes producing IL-6 and TNF-α (6). These studies demonstrate that VC has the role of immune-modulation. In this study, gene expression of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β were determined after macrophage infected by *M. bovis* for 3h and then treated with VC for 24h or 48h. The expression of three genes, IL-1β, IL-6 and TNF-α all increased significantly in the medium added VC group while IL-10 decreased relative to the control no matter at 24h or 48h, indicating that VC could selectively influence intracytoplasmic cytokine production of ANA-1 cells. However, the cytokine production of ANA-1 changed differently between the groups that cells infected with *M. bovis* (MB group) and the group that cells treated with VC post infection (MC group). Gene expressions of IL-1β and IL-6 were consistently higher in MC and MB groups at 24h and 48h than control. IL-1β continuously increased and the level of IL-1β in MC was higher than MB group. While IL-6 expression decreased from 24h to 48h in both groups. IL-1β and IL-6 could protect host cells from bacterial invasion. From this study, Macrophages treated with VC after infection with *M. bovis* are activated more than *M. bovis*-stimulated-only macrophages. VC could cause cells to secrete these two factors to help host against bacterial infection in the early stage. Unlike IL-1β and IL-6, TNF-α gene expression changed inconsistently in MC and MB groups. The level of TNF-α gene expression was lower in cells infected with *M. bovis* than control at 24h but increased from 24h to 48h and reached a higher level than control at 48h. Because the change of this gene was not detected before 24h, we can't know the tendency. We guess the production of this cytokine may rise and fall during the early 24h. Then inactivated cells were activated and the cytokine was reproduced and rose up again at 48h. It could be seen from Figure 4 that VC has played an important role in this gene expression modulation, because the gene expression of TNF-α in MC group was higher no matter at 24h or 48h than control although the trend of the expression level fell from 24h to 48h. IL-10 production, one member of Th2 type cytokines, is beneficial for *M. bovis* living in host cells (26). IL-10 down-regulates the Th1-type response and up-regulates Th2-type response in pathogen-host interactions, possibly leading to the lack of *M. bovis* host-protection as indicated by Jacobs et al (8). In humans and mice, increased IL-10 expression inhibits host restriction of intracellular M.tb growth (9,2). Here, IL-10 expression in all groups were lower than that of the control, and IL-10 expression in MB and MC groups were lower than VC medium group at 24h. This was consistent with the effects of VC on TNF-α, IL-6, and IL-1β with the bactericidal effect of the macrophages being stronger at 24h. But IL-10 gene expression in MB and MC group increased greatly at 48h; the MC group increase was significantly higher than that of the other groups. This finding may indicate that intracellular VC-exposed *M. bovis* enters NRP-like state with time, and develops microbiologically as it does *in vitro*. It may promote Th2 type cytokine IL-10 production and inhibit host cell immunity. In our study,

the results at 24h and 48h indicate that the cells' bactericidal ability is improved at early stage after VC added. Because the DosR regulon of *M. bovis* was not determined after infection with ANA-1, we can't assume when the gene was induced and the bacterial enter NRP. From the study by Taneja et al that VC triggered M.tb growth arrest and promotes dormancy phenotype development intracellularly in THP-1 cells (27). The difference between our study and theirs is the time duration of treatment with VC post infection. They treated with 2 mM AA ('AA') for 7 days post-infection. We cannot deduce whether the bacterial entered dormancy in our study, but we thought the bacteria was affected by VC also and the effect of VC on cell immunological function was an consequence of interaction with cell and bacterial together. From this point, further study need to elucidate the complicate interaction among the three factors.

Intracellular *M. bovis* growth was detected by bacterial enumeration. At 24h and 48h, the CFU of intracellular survival of *M. bovis* was lower in MC than in the MB group. This was consistent with the observation that *M. bovis* grows poorly in cultures with VC. VC induced growth arrest of *M. bovis in vitro* and inhibited intracellular bacteria growth.

Latent tuberculosis and M.tb persistence in cattle and humans remain major global health problems. Here, expression levels of 'dormancy regulon genes', morphological change, and growth arrest show that *M. bovis* may have entered a non-replication period quickly when *M. bovis* was exposed to VC *in vitro*. VC promotes the bactericidal ability of macrophages by immune-modulation of some cytokine production. This may provide a new way to find a method to control the disease using VC or other similar chemical compounds.

Acknowledgements

This work was supported by the key project of Ministry of Agriculture, China (Project no. 2009ZX08009-183B), the special scientific fund for non-profit public industry (Agriculture), China (Project no. 200903027), the Beijing Science Foundation of China (Project no. 6101002), and the Natural Science Foundation of China (Project no. 30972164).

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