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Short Communication

# Inhibition of Inducible Nitric Oxide Synthase Reactivates Growth of Dormant *Mycobacterium Tuberculosis* within Infected Macrophages

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**Abstract**

Tuberculosis is one of the top ten causes of human death. The generation of reactive nitrogen intermediates within the macrophages and to some extent in *Mycobacterium tuberculosis* cells as well is well documented. Release of NO from lung macrophage cells after challenge with *M. tuberculosis* bacilli is reported. However, the link between these reactive nitrogen intermediates and *M. tuberculosis* survival inside macrophage is still far from resolved. In this study, we have shown that supplementation of medium with nitrite (10mM) transformed the actively growing intracellular *M. tuberculosis* cells into dormant form. This is confirmed from colony forming units, acid fast staining and drug resistance studies. However, exposure of infected macrophage cells with *iNOS* inhibitor (1400 W) at 5μM concentration resulted in two-fold increase of intracellular growth of mycobacterial cells within 8 days of infection. Similar trend is also observed on nitrite exposed intracellular *M. tuberculosis* cells. Whereas 1400W does not have any significant effect on extracellular survival of either active or dormant *M. tuberculosis* cells. Therefore, this report suggests that both host NO and mycobacterial NO together play important role to achieve and maintain dormancy during intracellular stage. In addition, the inhibition of host derived nitric oxide could help to redesign the tuberculosis combination therapy..

## INTRODUCTION

*Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent of Tuberculosis (TB) discovered by Robert Koch in 1882. One-quarter of the population are infected with the latent form of *M. tuberculosis* [1]. The WHO has called for the end of TB by the year 2035 [2]. The major challenges of TB eradication are the increasing cases of drug-resistant bacilli, undiagnosed latent or dormant bacilli, and the length/cost of treatment [3]. *M. tuberculosis* infects lung macrophages, which serve as its primary host. Within the macrophage, *M. tuberculosis* can survive (as a latent or dormant form) or proliferate (active form) instead of being killed because of the capacity of *M. tuberculosis* cells to be modulated under oxidative stressed condition. Therefore, it is necessary to understand the role of redox system with greater detail on its survival inside alveolar macrophages.

Earlier reports have shown that macrophages exposed to *M. tuberculosis* bacilli induce $NOS_2$ gene expression and increase NO production [4], suggesting that host-derived NO could positively influence the conversion of actively growing bacilli into the dormant stage during Mtb pathogenesis and survival. Although the role of host NO in murine macrophages is well established as a protective agent against *M. tuberculosis*, it is not the same in humans. In addition, it has been suggested that immune-activated mouse macrophages produce 1,000 times more NO than human macrophages because of their difference in *iNOS* characteristics [5]. Recently, nitrite has been found to act as an inducer of mycobacterial dormancy even under aerobic conditions [6]. The nitrite reductase (NO forming) enzyme (*nirK*) converts nitrite into NO to influence this conversion [7]. However, the extent to which the NO produced by arginine and nitrite respectively and influence the *M. tuberculosis* bacilli life within the host is yet to be determined.

Therefore, in this report, using the red fluorescent reporter *M. tuberculosis* bacilli through the High Content Imaging tool, we have shown that nitrite induces dormancy of intracellular *M. tuberculosis* bacilli. Furthermore, NO produced by *iNOS* of host macrophages and nitrite reductase (*nirk*) from *M. tuberculosis* bacilli are together involved in the transition to dormancy. As a result, this report could provide a new approach towards the development of anti-tubercular chemotherapy, particularly against the latent form.

## MATERIAL AND METHODS

### Chemicals and Materials

Foetal Bovine Serum (FBS) and RPMI1640 obtained from

Invitrogen Life Technologies, Carlsbad, CA, USA. Dubos broth base and middle brook 7H11-agar base was purchased from BD Difco, USA. All other reagents were purchased from Sigma Aldrich otherwise mention. The stock solution was prepared in distilled water or Dimethyl Sulfoxide.

### Cell Lines and Bacterial Cultures

Human acute monocyte leukemia cell line ThP1, purchased from the National Center for Cell Science (NCCS; Pune, India), was maintained in a 25 or 75 $cm^2$ tissue culture flask (Eppendorf, Germany) containing RPMI cell culture medium with 10% heat-inactivated FBS. *M. tuberculosis* H37Ra RFP strain generated as described earlier [22] and grown in Dubos broth containing 50 µg/mL hygromycin under shaking condition at 120rpm at 37°C within a refrigerated shaking incubator (Model 431, Thermo electron Corporation, USA). For the macrophage infection, aerobic or log phase *M. tuberculosis* H37Ra RFP culture was filtered through an autoclaved 10-11 µm sterile Whatman filter before use [23].

### Growth Kinetics of *M. Tuberculosis* in Macrophage Cells in the Presence of Nitrite

Thp1 cells were infected with *M. tuberculosis* bacilli by following an earlier method [16]. Thp1 cells (~5 x $10^4$ cells/well) were seeded in the 96 sterile microplate (Corning, Sigma) containing 200-µL RPMI medium with 20nM of Phorbol Myristic Acetate (PMA). The plates were incubated at 37°C in the presence of 5% $CO_2$ and 95% humidity (Eppendorf, Germany) overnight to differentiate into a macrophage. Macrophage infected with *M. tuberculosis* H37Ra RFP at 1:1 MOI and incubated further for 10-12 h. After incubation, uninfected bacilli were removed by three times washing with sterile Phosphate Buffer Saline (PBS) followed by addition of 200 µL RPMI medium. Different concentration of nitrite (5, 10, 20, and 50mM) was added to the 96 microplate wells in triplicate. Plates were further incubated, and the average fluorescence spot count was periodically measured using XTI Array Scan (Thermo fisher,) as already described [23]. DAPI was used on the 8th day of experiment. Macrophage without nitrite acts as a vehicle control.

For the determination of colony count, the infected macrophages were lysed using 200 µL of hypotonic buffer pH 7.4 (10 mM HEPES buffer containing 1.5 mM $MgCl_2$ and 10 mM KCl) in the microplate wells. The 100 µL lysate was then spread on Middle brook 7H11 albumin agar plates to get the CFU after 3 to 4 weeks of incubation at 37°C.

### Intracellular Acid-Fast Staining

A modified Ziehl-Neelsen staining was used to detect the acid-fast property of intracellular *M. tuberculosis* by following an earlier established protocol [24-26]. On the 4th day of infection, medium was aspirated and was fixed with 4% paraformaldehyde (pH 7.4) for 10min, then washed with PBS. Primary stain carbolfuschin added to the staining well, incubated for 5 min. After incubation, washing was done with acid-alcohol for 3mins. Cells were counterstained with methylene blue for 4 mins. Finally, wells were washed with distilled water and dried the plate. Microscopic images were captured by EVOS microscope using 60X objective.

### Inhibition Kinetics of Standard Anti-Tubercular Drugs Against Nitrite Treated Intracellular *M. Tuberculosis*

Thp1 cells (~5 x $10^4$ cells/well) were seeded and infected as described above. Uninfected bacilli were removed by washing with PBS of pH 7.2, followed by the addition of 200 µL RPMI medium. Immediately after infection (day 0), 10mM of nitrite was added to the microplate and plates were incubated at 37°C in the presence of 5% $CO_2$ and 95% humidity (Eppendorf, Germany). After two days of incubation, standard anti-TB drugs such as Rifampicin (RIF), and Isoniazid (INH) at MIC concentration of 0.052, and 0.053, respectively were added [22]. Average red fluorescence spot intensity was periodically measured by using the XTI Array Scan (Thermo fisher).

### Effect of Inos Inhibitor on the Growth of Intracellular *M. Tuberculosis*

N-(3-(Aminomethyl) benzyl) acetamidine (1400W) is a specific inhibitor of *iNOS,* binds slowly and tightly to *iNOS* to develop a powerful inhibition [10]. In order to evaluate the effect of *iNOS* inhibitor (1400W) on the growth of intracellular *M. tuberculosis*, solution of two fold serially diluted 1400W inhibitor, at the concentration range (0-10µM), was added to the microplate in triplicate (after washing out the uninfected bacilli) containing infected *M. tuberculosis* with RPMI medium. The plates were incubated at 37°C in a $CO_2$ incubator with 95% humidity (Eppendorf, Germany). Fluorescence was periodically measured as described earlier [23]. Macrophages infected with *M. tuberculosis* without *iNOS* inhibitor acts as a vehicle control.

### Effect of Inos Inhibitor on the Growth of Nitrite Induced Dormant Intracellular *M. Tuberculosis*

Thp1 cells (~5 x $10^4$ cells/well) were seeded in the 96 well sterile microplate in 200 µL RPMI medium with 20nM of PMA. The plates were incubated at 37°C in the presence of 5% $CO_2$ and 95% humidity (Eppendorf, Germany) overnight to differentiate into a macrophage. After incubation, macrophages were infected with *M. tuberculosis* H37Ra RFP at 1:1 MOI and incubated further for 10-12 h. After PBS wash, RPMI media supplemented with only 10mM of nitrite or 10mM of nitrite along with 5µM concentration of *iNOS* inhibitor 1400W, in triplicate and plated were incubated at 37°C in the presence of 5% $CO_2$ and 95% humidity (Eppendorf, Germany). Growth was measured as fluorescence spot count as above.

## RESULTS

### Nitrite Induces Dormancy of *M. tuberculosis* Within the Macrophage

Nitrite treated intracellular *M. tuberculosis* exhibits growth

inhibition and reduced ATP consumption under hypoxic conditions [8]. However, there is no report on the same under normoxic conditions. Thus, to evaluate the effect of nitrite on the intracellular *M. tuberculosis*, its dose-dependent effect on the bacilli count was observed in infected macrophages (Figure 1A). It was found that there is no significant increase observed in *M. tuberculosis* spot count (1431.33 ± 305.31 on the 2nd day and the count 1015.01 ± 179.09 on the 8th day actually reduced) upon exposure to nitrite (10 mM). This result is corroborated with microscopy as well as Colony Forming Units (CFU) counts (Figure 1B & C). In fact, cell counts were found to increase up to 48h in both control and 10mM of nitrite treated infected macrophages (from 3.3 ± 0.10 to 3.7 ± 0.10 $\log_{10}$ CFU/mL). Afterwards, counts were significantly decreased with time in the nitrite-treated infected macrophages compared to control up to day 8 (5.43 ± 0.13 to 3.43 ± 0.12 $\log_{10}$ CFU/mL) (Figure 1B). A simultaneous DAPI staining on 8th day clearly indicates that the host cell remains very healthy (Figure 1C). The immediate reduction in the intracellular bacilli counts raised the possibility of developing acquired non-replicative features in them under the influence of nitrite in the medium.

### Dormancy Characteristics Developed in Nitrite Treated Intracellular *M. tuberculosis*

This shifting to a non-replicative dormant state of intracellular bacilli upon nitrite treatment was assessed by Ziehl-Neelsen staining (Figure 1E). The nitrite treated intracellular bacilli showed a pale blue stain, indicative of the loss of the acid-fast property.

Furthermore, it is known that the non-replicative dormant mycobacteria develop drug resistance characteristic [6,9]. We assessed the antibiotic resistance using RIF (an inhibitor which kills bacteria from Active or dormant stage) and INH (an inhibitor which kills only in actively growing stage), applying at their MIC concentrations, and observed that INH (1285.23 ± 263.32) did not show any significant inhibition on the nitrite treated intracellular bacilli (1553.05 ± 150.33) when applied on the 8th day of incubation except RIF (329.14 ± 85.42) (Figure 1F). These results indicated that nitrite-treated intracellular *M. tuberculosis* bacilli acquired dormancy characteristics.

### *iNOS* Inhibitor Exposure Induces Intracellular Growth of *M. tuberculosis* Bacilli

1400W is a specific inhibitor of *iNOS* without having any significant toxic effect to the mammalian cells [10]. Firstly, the dose response effect of 1400W inhibitor using a concentration range (0-10 μM) on the growth kinetics of *M. tuberculosis* is observed within infected macrophages (Figure 2A,B). The inhibitor when applied at > 2.5 μM concentration showed significantly increased level of red spot intensities ($p = 0.02$). The inhibitor effect reaches a plateau at 5 μM concentration. Therefore, we selected 5 μM as optimum concentration of 1400W inhibitor for further experiments. In order to check any potential toxicity on *M. tuberculosis* cells, 1400W was applied up to 50μM concentration and found to have no inhibitory effect on growth of the bacilli under *in vitro* conditions (Figure 2C). A parallel exposure of 1400W to extracellular *M. tuberculosis* cells under identical conditions found that the culture growth remained unaffected even at 50μM concentration of the inhibitor (Figure 2C). So. The effect of 1400W observed on intracellular *M. tuberculosis* bacilli is specific in nature.

### *iNOS* Inhibition Facilitate the Reactivation of Growth of Nitrite Induced Intracellular Dormant *M. tuberculosis*

As both macrophages and bacilli could produce NO through their *iNOS* and *nirK* enzymes respectively, we were interested to understand the extent of influence exerted either of these two in the bacterial physiology within infected macrophages. It becomes interesting to see the effect of *iNOS* (1400W) inhibitor on dormant intracellular *M. tuberculosis* bacilli in presence of nitrite. Therefore, we added 1400W inhibitor after 2 days of nitrite treatment and it was found that nitrite treated intracellular bacilli are showing significantly increased red fluorescence spot count (2317.89 ±228.79 on 6th and 2992.57±349.43 on 8th day) compared to only nitrite treated infected macrophage (1474.27±59.53 on 6th and 1555.74±196.32 on 8th day) respectively with time (Figure 3). This result indicated that the *iNOS* inhibitor (1400W) facilitate the reactivation of dormant intracellular bacilli even in presence of nitrite in the medium.

## DISCUSSION

Many of the earlier studies have clearly inferred that nitric oxide acts as an anti-tubercular agent [11]. Recently, it has been established that nitric oxide acts as an inducer of dormancy in *M. tuberculosis* cells [6]. It has been reported that *M. tuberculosis* bacilli could produce NO from nitrite and justifies the reason behind development of nitrite induced dormancy under aerobic condition [6,7]. So, iNOS of macrophage and *nir K* of *M. tuberculosis* bacilli together contribute to the total pool of NO produced within the macrophages. Earlier report suggested that nitrite inhibits the intracellular *M. tuberculosis* growth as well as reduced ATP consumption [8]. Nitric oxide in human is oxidized to produce nitrite and/or nitrate and finally excrete through urine [12]. This conversion depends on other environmental factors [13]. It is well known that NO is converted to nitrite in aqueous medium [14]. Most of the nitric oxide molecules released by activated macrophages are converted to nitrite even in absence of aqueous medium, when the alveolar macrophages are infected with *M. tuberculosis* bacilli in human lung, which was evidenced from analysis of sputum samples collected from TB patients [15]. A small part of this NO is converted to peroxynitrite after reaction with superoxide produced by *M. tuberculosis* bacilli which is finally rearranged to nitrate [16]. Anyways, nitrate could act as an alternate respiratory substrate under hypoxic conditions as well as an intracellular substrate for the entry of extracellular nitrite into the bacilli [16,17]. It could be considered that the entry of bacilli in the lung will trigger the release of NO to kill the bacilli in vain. Interestingly, we observed that external nitrite treatment also induced intracellularly growing bacilli to

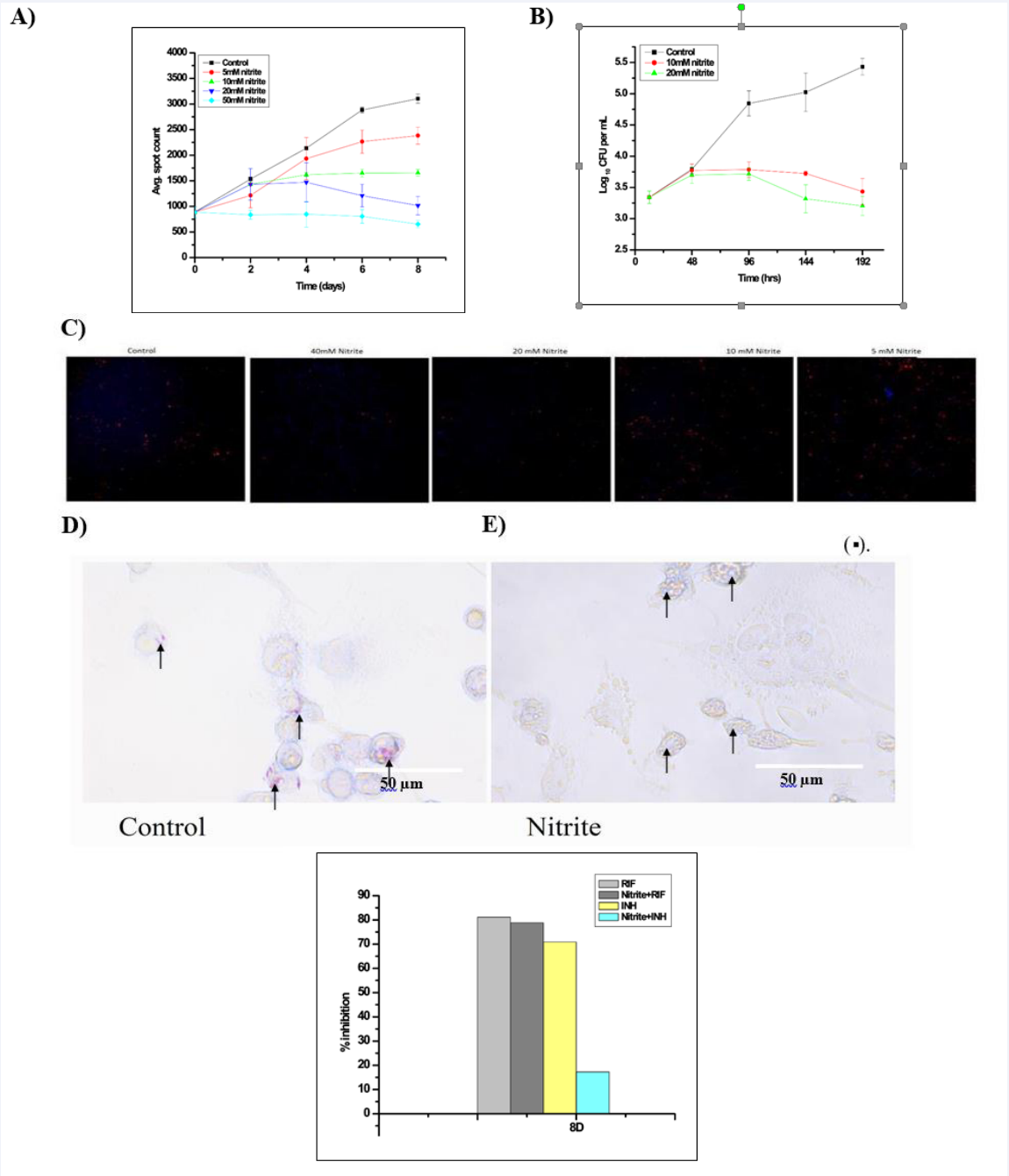


**Figure 1 Effect of nitrite on the growth of intracellular *Mycobacterium tuberculosis* : A) Nitrite Dose dependent growth of intracellular Mtb cells. The a**verage red spot intensity measured at different time (days), **B)** the average CFU count measured at different time points, **C) Intracellular fluorescent spot images**: Images were captures on 8th day after addition of DAPI. Each image represents one of the quadrants of the well. Periodically average red spot intensity was measured (HCA, Thermo-fisher) as described in "Material and Methods" section. The results are shown as the mean of three independent experiments ± standard error mean deviation (SEM). Macrophage without nitrite treated as a vehicle control. **Effect of nitrite exposure on the acid-fast property of intracellular *Mycobacterium tuberculosis* H37Ra RFP:** Intracellular *Mycobacterium tuberculosis* were stained with carbolfuschin and counter stained with methylene blue. D**)** Untreated Intracellular *Mycobacterium tuberculosis* were used as the control. **E)** Intracellular *Mycobacterium tuberculosis* cells treated with nitrite (10 mM). More details are described in the "Materials andMethods" section. Fluorescence microscopic images were captured at 60× objective using an EVOS microscope (Life Technologies). **F: Percentage inhibition of standard anti-tubercular drugs against intracellular *Mycobacterium tuberculosis*:** Control and nitrite treated intracellular MtbH37Ra RFP was treated with drug like RIF (0.05µg/mL), and INZ (0.53µg/mL). Percentinhibition was calculated on 8th day of drug treatment. Rest details are mentioned in "Materials and Methods". % inhibition = (vehicle control-test/vehicle control-blank) *100 Nitrite treated with drug as a test, and nitrite act as a vehicle control.

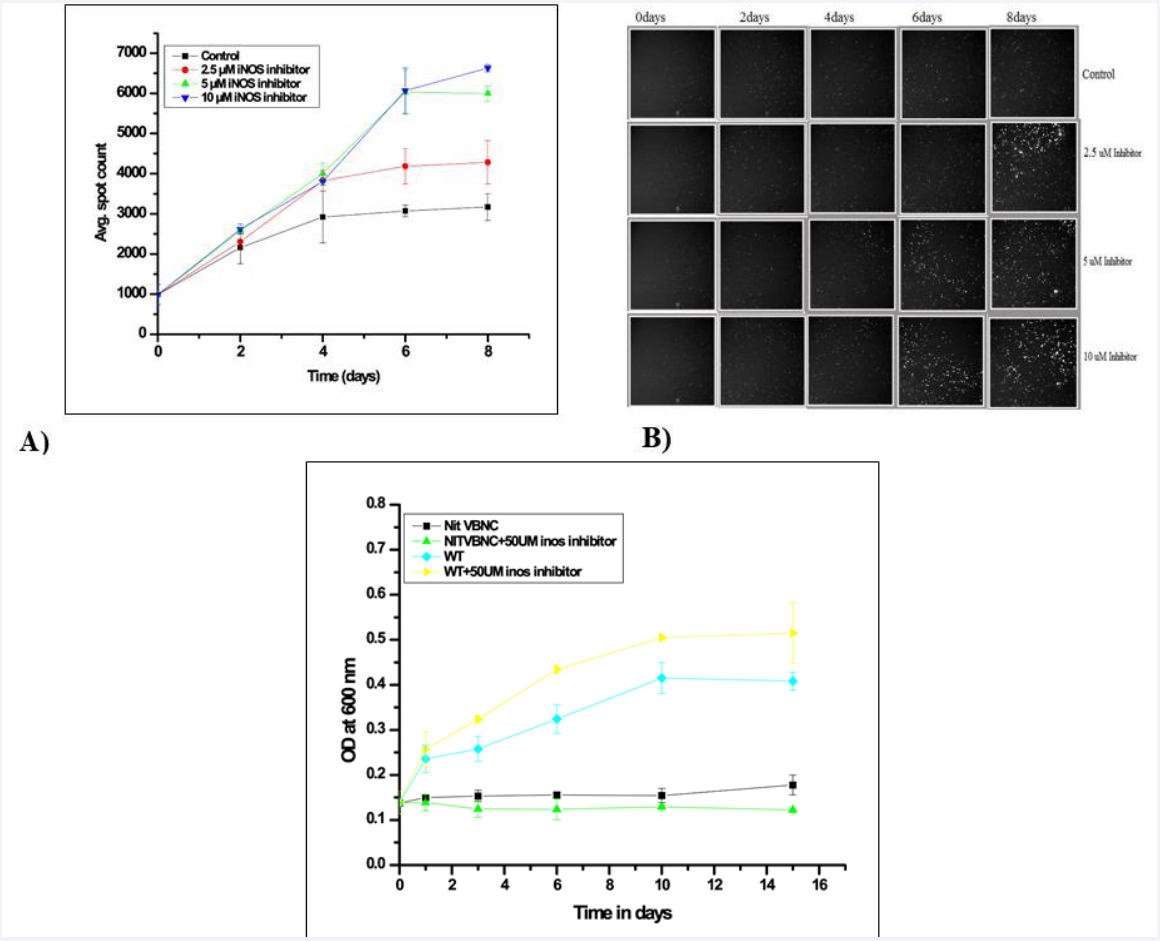


**Figure 2 Effect of 1400 W on the growth of intracellular *Mycobacterium tuberculosis:*** Different concentration of *iNOS* inhibitor (1400W) (2.5, 5, 10 μM) was added in each well after removal of extracellular bacilli and further incubated at 37 °C, 5% $CO_2$ atmosphere. Periodically average red spot intensities were measured (HCA, Thermo-fisher) as explained in"Material and Methods" section. The data represent as means of the triplicate results with ± SD. **B) Intracellular fluorescent spot images**: Images were captures during the read and *processed using Cellomics software. Each image represents one of the quadrants of the well.* **C: Effect of 1400 W on the growth of extracellular *Mycobacterium tuberculosis*** 1400W was added at 50 μM concentration in each triplicate well of VBNC and wild type Mtb culture and further incubated at 37 °C. Periodically optical density were measured as explained in "Material and Methods" section. The data represent as means of the triplicate results with ± SD.

non-replicative dormant stage within the macrophages (Figure 1). Furthermore, acquired characteristic features (loss of acid fastness and antibiotics resistance) also confirmed that nitrite induced dormancy is achieved in the bacilli [18,9]. In our study, we have shown that nitrite treated intracellular bacilli also lose acid fastness characteristic within 2 days of nitrite exposure (Figure 1D,E), otherwise it takes almost 8 days after infection. So, the faster induction of actively growing bacilli to non-replicating stage depends among others, on the concentration of nitrite within the host milieu and bacilli could avail this option as an opportunity to attain early development of dormancy to survive within the host. The resistance to INZ treatment and susceptibility to RIF indicated that intracellular bacilli (Figure 1F) maintained a dormant state throughout the experiment. So, the routes of host NO go to nitrite within the TB infected human lung to make sure that the bacilli develop and maintain latency.

Nevertheless, *iNOS*/NO production is an important component of the host defence strategy against any infection. Herein, we observed that inhibition of host *iNOS* enhances the growth of intracellular *M. tuberculosis* bacilli (Figure 2). Earlier report also suggests that *iNOS* induced in murine macrophage due to the infection of *M. tuberculosis* and protected the host whereas avoidance of NO is needed for *M. tuberculosis* survival [19-21]. Martin, *et al* also reported that the NO exposure to *M. tuberculosis* under *in vitro* culture conditions induces dormancy via up regulation of the dormancy regulon genes [17]. Interestingly, our robust experimental conditions allow controlled manipulation of the *iNOS/nirK* function to understand their precise influence on the intracellular bacilli. The application of *iNOS* inhibitor is also found to break the nitrite induced dormancy of intracellular bacilli (Figure 3). This clearly indicated that intervention of host NO stress could lead to the reactivation of the dormant

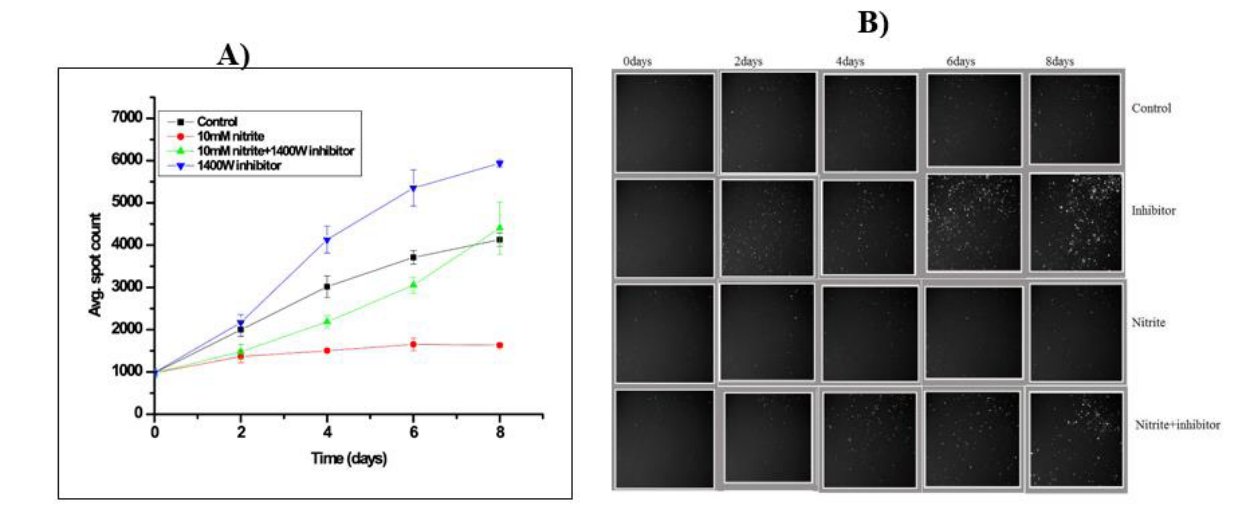


**Figure 3 Effect of *iNOS* inhibitor on growth of nitrite induced intracellular *dormant Mycobacterium tuberculosis* bacilli**: Macrophages were infected with actively growing Mtb H37Ra RFP cultures with ~1:1 MOI. After 10h of incubation extracellular bacilli were washed with 1X PBS for 3 times, followed by addition of 10mM of nitrite, 10mM of nitrite along with 5 µM 1400 W, 5 µM *1400W* only was added in each triplicate well. The plate was further incubated at 37 $^{O}$C, 5% $CO_2$ atmosphere. **A)** Periodically red spots intensities were measured (HCA, Thermo-fisher) as explained in "Material and Methods" section. The data represent as means of the triplicate results with ± SD. **B) Intracellular fluorescent spot images**: Images were captures during the read and processed using Cellomics software. Each image represents one of the quadrants of the well.

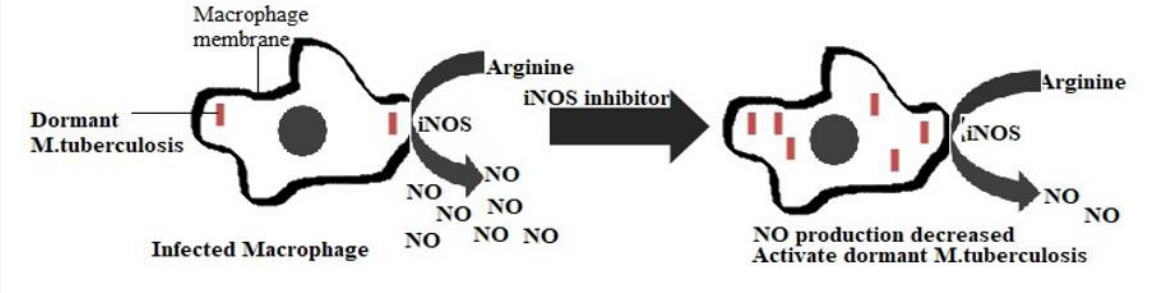


**Figure 4 Graphical representation of reactivation of dormant *Mycobacterium tuberculosis:*** In macrophage, iNOS induces NO production from arginine as a substrate. NO of both the mycobacteria and host macrophage system helps to maintain dormancy characteristics of *M. tuberculosis* within the infected macrophage. Upon inhibition of host*iNOS using* 1400W inhibitor dormant mycobacteria starts replicating.

intracellular bacilli even in presence of nitrite. As, host *ions*' is the source of total nitrite pool in the lung, *iNOS* inhibitor lead to the decrease in nitrite dependent NO production in lungs to destabilize the maintenance of non-replicating status of the bacilli. Graphical representation of (Figure 4) shows the reactivation of *M. tuberculosis* with inhibition of host NO production.

In summary, nitrite induced dormancy of intracellular *M. tuberculosis* cells is confirmed by CFU, loss of acid fastness, and drug resistance towards INH. Also, inhibition of macrophage NO production regained the active state of *M. tuberculosis* cells inside the macrophages. This finding could be useful to redesign and shorten the therapeutic treatment against TB.

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### Author Contributions

The study was carried out by SA. SA wrote the original

draft and AY helped to analyze the data. Overall supervision is provided by DS.

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