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# Assessment of phagosomes infected with *Mycobacterium tuberculosis* as a vaccine candidate against tuberculosis

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The present study describes a novel and simple vaccination strategy that involve culturing of *M. tuberculosis* in the macrophage cells. Isolation of phagosome from macrophage (cell line J774) infected with *M. tuberculosis* (H37) and *M. bovis* (BCG) at early and late phase of infection was done ensuing the identification and characterization of these phagosome. *In vitro* study of apoptosis induced by phagosome infected with (H37) and (BCG) was performed. The vaccine candidate with H37 MOI- 1:10 at 3 h, MOI- 1:20 at 1, 1.5, 2.5 and 3 h and BCG MOI- 1:20 at 3.5 h showed percentage apoptosis as 38.64, 39.93, 34.66, 22.56,34.59 and 37.81% respectively. The results designates that macrophages provide cellular niche during infection and illustrate considerable immunogenic property. Novel antigens expressed or secreted by H37 in infected macrophages can provide evidence to be a successful vaccine candidate as it endures enhanced immune response than BCG.

**Keywords:** Apoptosis, BCG, Cell line, Mycobacterium tuberculosis, Vaccine

Around the world, tuberculosis (TB) was one of the leading cause of death in 2012; 8.6 million people fell ill with TB and 1.3 million people died[1]. India is one of the high burden countries for tuberculosis as well as drug-resistant tuberculosis. As per WHO, estimated number of new tuberculosis cases in India, from 2000 to 2012 has increased to 825629[2]. Being the most prominent infectious agent, *Mycobacterium* is estimated to infect 90 million people and kills 30 million people[3,4], which has now increased to 9.2 million new tuberculosis cases and approximately 2 million tuberculosis- related deaths in this decade[5]. Bacillus Calmette Guerin (BCG); live, attenuated *M. bovis* is the only vaccine available till date. However the uncertain efficacy of BCG in the prevention of pulmonary TB in adult[6,7] and the emergence of extensively drug resistance *M. tuberculosis* strain has further enhanced the sense of urgency in the development of new and effective TB vaccine against all populations. World health organization has stopped recommending utilization of BCG vaccine for infants at high risk of HIV infection[8]. Natural *M. tuberculosis* interaction with an individual provide resistance towards TB thus a future vaccine is desirable which provide immunity to individual with latent infection and could provide protection as given by natural interaction[9].

Alveolar macrophages are the primary cell type which firstly interacts with *M. tuberculosis*[10]. Apoptosis may constitute another effectors mechanism for the infected host to limit outgrowth of *M. tuberculosis*. Apoptosis of phagocytic cell may prevent dissemination of infection. The interplay between *M. tuberculosis* and human host determine the outcome after infection[11]. Macrophages thus have the capability to process *M. tuberculosis* antigen to trigger an immune response. *M. tuberculosis* has been found to sequester within the phagosome of macrophage and has the ability to survive within macrophages[4].

Since mycobacterial load is reduced but complete sterility is not provided by the BCG, as its immune response is weak and it is also unable to stimulate combination of T-cell thus there is a need for a more effective vaccine. A vaccine with viable *M. tuberculosis* can be a light to the problem as it results in the activation of various T-cell subpopulations which in turn helps to achieve better immune response[12]. Therefore the vaccine with such a concept should be introduced which gives protection against latent infection and also give a long term

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protection. More effective vaccine and new therapeutic strategies are needed which not only benefit the individual who is infected but also limit the spread of infection[9].

Designing a novel rational vaccine against TB is based on the assumption that few selected antigenic peptide identified by CD4 T-cell in context to MHC1 are sufficient for protective immune response. It also depends upon the assumption that as many antigens as possible should be present to activate a larger repertoire of T cell population comprising conventional CD4 and CD8 T Cell[13]. Thus, priorities in fundamental research in vaccine development should aim at determining the component of host immune system that are critical for control and elimination of bacteria. This will involve determining the respective role of innate and adaptive immunity in preventing *M. tuberculosis* infection. The vaccine preparation also depend upon reactivation of latent disease and better understanding of immune response against metabolic stages of pathogen and in different population involving HIV infected person, person of various ages. The vaccine candidate introduced in this study depends upon: internalization of macrophage, *M. tuberculosis* is enclosed in a specified vacuole; the phagosome. During maturation of phagosome, various destructive mechanisms lead to degradation of *M. tuberculosis*. However, *M. tuberculosis* possess the capacity to modulate their own trafficking route by inhibiting phagosome-lysosome fusion ant to create an intracellular niche allowing them to survive for prolonged periods inside the host[14]. *M. tuberculosis* generates better immune response against tuberculosis and long lasting memory response to combat tuberculosis. Thus the following objective is proposed to access the vaccine candidate against tuberculosis prepared from *M. tuberculosis* infected phagosomes.

## Materials and Methods

*Culture medium*—Lowenstein Jensen (LJ) Medium was purchased from Hi-Media and was prepared by following the method of Vanketaramen and Paramsivan[15].

*Mineral salt solution shake*—The mineral salt solution containing following components (g/600 mL) -potato starch soluble (30), L-asparagines (3.6), monopostassium phosphate (2.4), magnesium citrate (0.60) and magnesium sulphate (0.24) was hydrolyzed and autoclaved at 121 °C and 15 lbs for 15 min.

*Malachite green solution*—Malachite green solution (2%) was prepared and sterilized by autoclaving at 121 °C and 15 lbs pressure for 15 min.

*Preparation of complete medium*— Fresh eggs (hen) were procured and cleaned by scrubbing with hand brush in 5% soap solution. Eggs were thoroughly rinsed in running tap water and soaked in 70% alcohol for 15 min. They were broken with sterile knife poured into a sterile flask containing glass beads and thoroughly homogenized. The homogenized egg suspension was filtered through four layered sterile gauze into beaker. The mineral salt solution with Malachite green (600 mL) and homogenized egg suspension (1000 mL) were aseptically pooled in large sterile flask and mixed well.

Medium (10 mL) was poured into each sterile McCartney bottle. The screw capped bottles were kept in slanted position in an inspissator. The medium was coagulated in this way by inspissation at 85 °C for 1 h for two consecutive days. The sterility of the suspension was confirmed after inspissation; L-J media following incubation at room temperature for 1 day and then stored at 4 °C for further study. Middle brook 7H9 broth was purchased from Hi-Media and was prepared following the method of Singh *et al.*[7]. Middle brook 7H9 broth base [containing following components (g/L) ammonium sulphate (0.50), disodium phosphate (2.50), monopotassium phosphate (1), sodium citrate (0.10), magnesium sulphate (0.05), calcium chloride (0.0005), zinc sulphate (0.001), copper sulphate (0.001), ferric ammonium citrate (0.04), biotin (0.0005) and malachite green (0.001)] – (4.7 g) was dissolved in 900 mL de-ionized water containing 2 mL of glycerol and sterilized by autoclaving at 121°C and 15 lbs. pressure for 15 min. The medium was cooled at room temperature and then aseptically 100 mL of albumin dextrose catalase (ADC) enrichment supplement was added to the medium. The medium was stored at 4 °C until further use.

*Bacterial cultures—Mycobacterium tuberculosis* H37 (ATCC 8933) and *Mycobacterium bovis* BCG (Danish Strain) were procured from National Jalma Institute of Leprosy & Other Mycobacterial Diseases, Agra.

*Growth of M. tuberculosis H37 and BCG*—Cultures were grown in 7H9 medium containing 0.05% Tween-80 supplemented with 10% albumin dextrose catalase (ADC) enrichment supplement. The culture flasks were kept in sealed container for the

growth of *M. tuberculosis.* Oxygen present in flask was utilized by bacteria in approximately one week and after which stressed condition was created as culture was incubated for 42 days in Orbital Shaker incubator at 170 rpm at 37 °C.

*Cell line*—Macrophages cell line (J-774) was procured from Immunology Division, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India.

*Establishment of cell line*—Establishment of cell line was done following the method of Sharma and Agrewala[16]. Macrophage cell line (J-774) was plated in RPMI-1640 medium [supplemented with sodium pyruvate (1mmol/L), L-glutamine (2 mmol/L), penicillin (75 µg/mL), streptomycin (100 µg/mL), 2-β mercaptoethanol (50 µmol/L) and HEPES (10 mmol/L)] and were grown in $CO_2$ incubator (Heal force$^R$, Lishen) at 37 °C at 5% $CO_2$.

*Mcfarland standard solutions*—Mcfarland nephelometer barium sulphate standard was prepared following the method of McFarland[17]. McFarland standard represents specific concentrations of CFU /mL and is designed to estimate number of cells per mL.

*Culturing of bacteria in macrophages*— Infection of macrophage was done by following the method of Sharma and Agrewala[16] with certain modification including 5% $CO_2$ concentration instead of 7% and difference in time interval for incubation of cell line in antibiotics. Briefly, cell line was infected with *M. tuberculosis* (H37) and *M. bovis* (BCG) at an MOI of 10 and 20 bacteria/macrophages. The cells were then treated with Amikacin (50 µg/mL) and Isoniazid (100 µg/mL) at an interval of 30 min, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 h, after 72 h of incubation extensive washing with RPMI-1640 medium were carried out to removes the unbound cells. The cells were then harvested by 0.5 M EDTA solution and centrifuged at 300 g for 5 min at 4 ºC supernatant was discarded and pellet was resuspended in phosphate buffer saline (PBS).

*Phagosome identification*—Identification of phagosomes infected with *M. tuberculosis* (H37) and *M. bovis* (BCG) was done following the method of Armstrong and Hart[18] and Torres *et al* [19]. Briefly, cells (bacteria cultured in macrophage) were incubated at 37 °C for 10 min and washed in ice-cold RPMI to remove extracellular bacteria. Prewarmed medium was added, and cells were incubated at 37 °C for up to 4 h. Cells were fixed with 1% paraformaldehyde and washed. After 24 h, supernatants (100 µL) were harvested. Uptake of live *M. tuberculosis* by macrophage cell line J774 was assessed by identification of acid-fast bacilli with Ziehl-Neelsen staining of cells. Three hundred cells were analyzed to determine the number of cells that had phagocytized *M. tuberculosis*. Morphological characters of cell line were done by observing the culture plate under 100X Microscope and to observe the infection rate of the pathogen acid fast staining was carried out.

*Phagosome characterization*—To characterize the phagosomes infected with *M. tuberculosis* (H37) and *M. bovis* (BCG) DNA laddering was performed by following the method of MacGurn and Cox[20] and Kashibhatla *et al.* [21] with certain modification, that is 3 µL RNase and 3 µL protease K was used. Briefly, macrophages were seeded onto 10 cm tissue culture dishes containing sterile glass coverslips. *M. tuberculosis* cultures were washed three times in phosphate-buffered saline (PBS), centrifuged at low speed to remove large clumps. Inoculum was prepared by diluting bacteria to the required multiplicity of infection (MOI) of 10 and 20 RPMI medium. Macrophage monolayers were inoculated, washed three times with warm PBS, covered in warm RPMI medium, and returned to the incubator. J774 cells were plated on 96 well microtitre plate infected with *M. tuberculosis* (H37) and *M. bovis* (BCG). Cells were harvested with 0.5 EDTA and were centrifuged at 2500 rpm for 5 min and resuspended in 40 µL citrate phosphate buffer. Cells were then centrifuged at 5000 rpm for 5 min and the supernatant collected after transferring to new tube with 3 µL of RNAse was added and then sample was incubated at 37 °C for 30 min. Proteinase –K (3 µL) was then added and sample was incubated for 10 min at 37 °C. Agarose gel electrophoresis was carried out by mixing sample in 12 µL of loading buffer and was loaded on 1.5% agarose gel. After electrophoresis, gel was observed in Transilluminator (Biotech, India).

*In vitro estimation of apoptosis induced by phagosome infected with M. tuberculosis (H37) and M. bovis (BCG)*—Rezazurin assay was performed to check the apoptosis induced by phagosome by following the method of Palomino *et al*[22]. Macrophage cell line J774 were cultured in 96-well flat-bottom plates ($1.5 \times 10^5$ cells/well) in RPMI 1640 medium and phagosome that is J774 cells infected with *M. tuberculosis* (H37) and *M. bovis* (BCG)

were placed on these 96 well microtitre plate. 30 µL of 0.02% dye was added to each well and were incubated. Media was removed carefully without disturbing the cells. Cells were then agitated on orbital shaker for 15 min and absorbance was noted at 610 nm.

## Results

*Growth of M. tuberculosis on LJ medium and middle brook 7H9 medium—M. tuberculosis* was observed as cream white dry rough colonies with wrinkled surface initially, which turned to yellowish or buff coloured latter. Bacilli growth in liquid medium appeared on surface, prominent pellicle like structure was observed throughout the medium.

*Culturing of M. tuberculosis* (H37) and *M. bovis* (BCG) *in macrophages cell line J-774*—$1\times10^6$ J-774 macrophages were infected with a multiplicity of infection (MOI) of 1:10 and 1:20 of *M. tuberculosis* H37 and *M. bovis* BCG. Infected macrophages were observed by acid fast staining (Table 1). *M. tuberculosis* H37 at MOI of 1:10 was found to be positive after 3 h of infection whereas during MOI of 1:20, *M. tuberculosis* H37 were found to be positive at 0.5, 1.5 and 3 h. *M. bovis* BCG were found to be positive only after 3 h at MOI of 1:10 (Fig.1).

*Induction of DNA fragmentation by a vaccine candidate*—The entire vaccine candidates tested against the macrophages to induce the cell death by DNA laddering resulted in fragmentation. Macrophages infected with *M. tuberculosis* H37 and BCG did not induced the DNA fragmentation of the macrophages.

*In vitro estimation of apoptosis induced by phagosome*—Table 2 represents the macrophage cells viability against the phagosome, Macrophages infected with *M. tuberculosis* and *M. bovis* at MOI of 1:10 and 1:20 at various interval of time. *M. tuberculosis* H37 (MOI, 1:10) have shown minimum viability at 1, 1.5, 3 and 3.5 h of infection against macrophages. BCG (MOI, 1:10) have shown minimum viability at 1.5 h of infection. BCG (MOI, 1:20) have shown maximum growth at 30 min after infection.

*Reduction of J774 cells by phagosome*—Percent reduction in the growth of J774 cells induced by phagosome, macrophages infected with *M. tuberculosis* H37 (MOI, 1:10 and 1:20) and *M. bovis* (MOI, 1:10 and 1:20) were determined. *M. tuberculosis* H37 (MOI 1:20) reduced the growth by 38.64, 39.93, 34.66 and 22.56% at 1, 1.5, 2.5 and 3 h respectively. *M. tuberculosis* H37 (MOI, 1:10) have reduced the growth by 27.64% and 26.62% at 1 h and 3 h respectively. BCG (MOI, 1:20) has reduced the growth at 3.5 h and BCG (MOI, 1:10) reduced the growth of macrophages by 34.59% at 4 h (Table 3).

## Discussion

The search for an effective vaccine against tuberculosis has been ongoing for well over 100 years, yet a successful candidate has been elusive[23]. The protective efficacy of current *M. bovis* BCG vaccine is both unpredictable and highly variable[24]. Therefore, continuous efforts are being made worldwide to prosper effective vaccine against tuberculosis.

Many researchers have conducted an assortment of studies with diverse antigens of *M. tuberculosis,* but their antigens have elicited only partial protection[25]. DNA vaccination studies have revealed an association with the elicitation of CD8 and CD4 cellular

Table 1—Acid Fast staining of J774 Macrophages cell line infected with *M. tuberculosis* and *M. bovis* BCG at different time periods

| Sr. no | Time (in hours) | J 774 Macrophages cell line infection | | | |
|---|---|---|---|---|---|
| | | *M. tuberculosis* H37 (MOI 1:10) | *M. tuberculosis* H37 (MOI 1:20) | *M. bovis* BCG (MOI 1:10) | *M. bovis* BCG (MOI 1:20) |
| 1 | 0.5 | nil | + | nil | nil |
| 2. | 1 | nil | nil | nil | nil |
| 3. | 1.5 | nil | + | nil | nil |
| 4. | 2 | nil | nil | nil | - |
| 5. | 2.5 | - | nil | - | - |
| 6. | 3 | + | + | + | nil |
| 7. | 3.5 | + | nil | nil | nil |
| 8. | 4 | - | nil | nil | - |

*+ = AFB positive, nil = AFB negative and - = not determined*

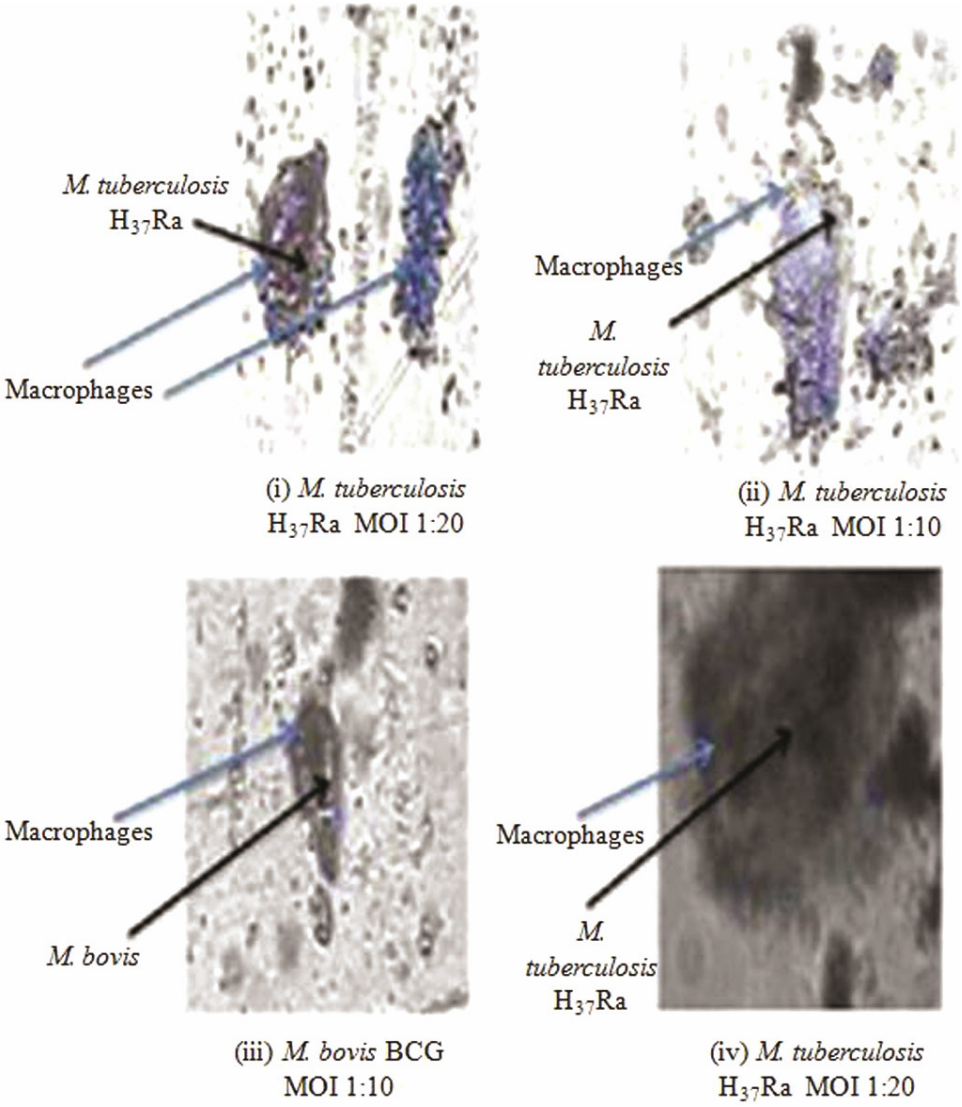


Fig. 1—*M.tuberculosis* engulfed in J774 macrophages cell line (i) *M. tuberculosis* $H_{37}Ra$ at 1:20 MOI after 1.5 hr, (ii) *M. tuberculosis* $H_{37}Ra$ at 1:10 MOI after 3.5 hr, (iii) *M. bovis* BCG at 1:10 MOI after 3 hr, (iv) *M. tuberculosis* $H_{37}Ra$ at 1:20 MOI after 3 hr. Blue arrow indicates the macrophage cell of cell line J774 and black arrow indicates the *M. tuberculosis* engulfed in inside cell line

Table 2—*In vitro* assay to check the apoptosis induced by the Macrophages infected *M. tuberculosis* H37and *M. bovis* BCG spectrophotomertically at 610 nm

| Time (in Hours) | *M. bovis* BCG (MOI, 1:10) | *M. bovis* BCG (MOI, 1:20) | *M. tuberculosis* H37 (MOI, 1:10) | *M. tuberculosis* $H_{37}$ Ra (MOI, 1:20) |
|---|---|---|---|---|
| Blank | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.5 | 0.868 | 1.219 | 0.951 | 0.908 |
| 1 | 0.828 | - | 0.783 | 0.664 |
| 1.5 | 0.600 | 0.789 | 0.902 | 0.650 |
| 2 | 0.824 | 0.939 | - | 0.886 |
| 2.5 | - | - | - | 0.707 |
| 3 | 0.847 | - | 0.794 | 0.838 |
| 3.5 | 0.797 | 0.678 | 0.869 | - |
| 4 | 0.708 | 1.269 | - | 0.959 |

*- = not determined*

immunity as well as humoral immunity, but the loom has not yet shown any encouraging result towards the development of viable vaccine[26]. Further, many facts recommended that secreted or soluble antigen of *M. tuberculosis* are probably target of protective immune response, but none of the vaccine candidate isolated from *Mycobacteria* grown in *in vitro* cultures are known to induce better protective immunity and generation of memory T-cells than BCG. Therefore, this evidently indicates that the protective antigens of *M. tuberculosis* that can be effectively exploited as vaccine candidate still need to be isolated. Sharma and Agrewala[16] described a novel and simple vaccination strategy that involve the culturing of live *M. tuberculosis* in macrophages followed by drug treatment and gamma irradiation, to kill the bacteria. Notably, the bacteria cultured in macrophages showed better protection, compared with the free bacteria, which indicates that the novel antigens expressed or secreted by *M. tuberculosis* in the infected macrophages may be of utmost importance as a vaccine candidate and need to be isolated and characterized.

The present study was conducted to examine whether a phagosome prepared by culturing *M. tuberculosis* H37 and *M. bovis* BCG in J774 macrophages cells generate any effective immune response against tuberculosis. The vaccines were prepared by culturing the bacteria at MOI of 1:20 and 1:10 at various intervals of time, so as to allow the macrophages for pathogen engulfment.

According to Filion *et al.* [23] *M. tuberculosis* has several mechanisms to abate killing at various stages of the infection. Potential options may exist to subvert the infection by it by focusing and altering the immune response to this pathogen at the different stages of the disease process. BCG has botched to protect against adult forms of tuberculosis in developing countries[27], so there is an urgent need to understand the differences in the pathogenesis of tuberculosis. *M. tuberculosis* inhibits clinical Th-1 mediated activation, because it blocks phagosome maturation[28], lysosome fusion, presentation via MHC-I and triggering via the IFN-γ receptor[29]. Therefore, during the present study, we have used a novel strategy was used to assess a vaccine candidate, i.e. phagosome, by culturing live *M. tuberculosis* H37 and *M. bovis* BCG, in macrophages, thus providing conditions for bacteria to row in natural environments. The preparation was made safe for use by killing the bacteria by drugs i.e. amikacin and isoniazid.

Macrophages infected with *M. tuberculosis* H37 (MOI, 1:20) at 3.5 h, *M. tuberculosis* H37 (MOI,1:10) at 2, 2.5 and 4 h, *M. bovis* BCG (MOI, 1:10) at 2.5 h and *M. bovis* BCG (MOI, 1:20) at 1, 2.5 and 3 h were not analyzed because the sample were lost during the preparation of phagosome. Rest of the phagosomes containing *M. tuberculosis* H37 and BCG were analyzed for pathogen engulfment by acid fast staining of the vaccine candidates prepared. Macrophages infected with *M. tuberculosis* H37 at MOI of 1:10 after 3 and 3.5 h and at MOI of 1:20 after 30 min, 1.5 h and 3 h were found to be positive for AFB staining. *M. bovis* was found to be positive only on 3 h at MOI of 1:10 while all the candidates were found to be negative at MOI of 1:20.

Sharma and Agrewala[16] described a novel and simple vaccination strategy that involves the culturing of live *M. tuberculosis* in macrophages followed by drug treatment and gamma irradiation, to kill the bacteria. Notably, the bacteria cultured in

Table 3—Percentage reduction of J-774 cells by macrophage infected *M. tuberculosis* H37 and *M. bovis* BCG

| Time (in Hours) | *M. bovis* BCG (MOI, 1:10) | *M. bovis* BCG (MOI, 1:20) | *M. tuberculosis* H37 (MOI, 1:10) | *M. tuberculosis* H37 (MOI, 1:20) |
|---|---|---|---|---|
| Blank | 0.00 | 0.00 | 0.00 | 0.00 |
| 0.5 | 80.22 | 100 | 87 | 83.9 |
| 1 | 76 | - | 72.36 | 61.36 |
| 1.5 | 64 | 72.9 | 83.36 | 60.07 |
| 2 | 76.1 | 86.76 | - | 81.88 |
| 2.5 | - | - | - | 65.34 |
| 3 | 78.2 | - | 73.78 | 77.44 |
| 3.5 | 73.65 | 62.19 | 80.31 | - |
| 4 | 65.4 | 100 | - | 88.63 |

*- = not determined*

macrophages showed better protection, compared with the free bacteria, which indicates that the novel antigens expressed or secreted by *M. tuberculosis* in the infected macrophages may be of utmost importance as a vaccine candidate and need to be isolated and characterized.

DNA fragmentation is considered to be a biochemical hallmark of apoptosis, where the DNA cleavage is believed to be an endogenous $Ca^{2+}$ and $Mg^{2+}$ dependent endonuclease able to break double stranded at internucleosomal sites[30]. The entire vaccine candidate tested against the J774 macrophages was found to be negative to induce apoptosis followed by DNA fragmentation, which indicates that the vaccine candidate have not initiated the endonuclease mediated cleavage of nuclear DNA. Over the last two decades, much has been learned about the immune response to Mtb both ineffective clearances of the pathogen and in the development of disease, which often continue unabated[31].

Apoptosis, also referred to as programmed cell death, is a process that is important to the development and maintenance of the immune systems[32]. Macrophage apoptosis may contribute to the innate immune system response against *M. tuberculosis* by containing and limiting the growth of tubercle bacilli. During the course of study, vaccine candidate, *M. tuberculosis* H37 (MOI, 1:20) at 1, 1.5, 2.5 and 3 h caused apoptosis by reducing the growth of macrophages cell line by 38.64, 39.93, 34.66 and 22.56% respectively. Vaccine candidate prepared by infecting J774 cells by *M. bovis* (MOI, 1:10) at 4 h and *M. bovis* (MOI, 1:20) at 3.5 h caused apoptosis by reducing the growth of macrophages cell line by 34.59 and 37.81% respectively, while, other vaccine candidates have not reduced the growth. Thus, a vaccine candidate prepared by Macrophages infected with *M. tuberculosis* H37 (MOI. 1:10) at 3 h, *M. tuberculosis* H37 (MOI. 1:20) at 1, 1.5, 2.5 and 3 h, BCG (MOI, 1:20) at 3.5 h can be used as a potential vaccine candidate to endure better immune response and protection against the tubercle infection. Macrophages are commonly regarded as the phagocytic cells that initially ingest *M. tuberculosis*, and provide an important cellular niche during infection. Macrophages are also considered to be important effector cells of the cellular immune response in tuberculosis. Therefore, Macrophages infected with tubercle bacilli can endure better immune response and can be a good vaccine candidate.

The present study indicates that the phagosome, macrophages infected with *M. tuberculosis* H37 has immunogenic properties and it could be used for effective anti-TB immune therapy; which indicates that the novel antigens expressed or secreted by *M. tuberculosis* H37 in the infected macrophages may be of utmost importance and might well prove successful as a vaccine candidate to endure better immune response.

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