The effect of repeated BCG Immunization in Mice challenged with *Mycobacterium tuberculosis*

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Abstract

Background: Tuberculosis (TB) is one of the most prevalent cause of death due to a single pathogen. Bacillus Calmette Guérin (BCG) is the only vaccine available for clinical use that protects against miliary TB; however, this vaccine has shown variable levels of efficacy against pulmonary TB. In Sudan, a single dose of BCG vaccine is given and there are few countries where repeated doses of BCG are given. The incidence of TB in Sudan is very high inspite of primary vaccination in neonatal period and therefore requires consideration for repeated immunization.

Objective: To investigate the effect of repeated BCG immunization in Mice challenged with M. tuberculosis.

Materials and Methods: A total number of 56 mice were examined for their immunopotency and protective efficacy of BCG against challenge dose of *M. tuberculosis* as single and second repeated dose. In experiment contains three groups of mice each of ten mice n = 10 (30mice). The first group(A) were immunized with BCG (0.2ml) first dose (I.P) for 21 days. The second group (B) were immunized with BCG (I.P) as first dose for 15 days and boosting dose (second dose) for another 15 days. The third group(C) were not immunized with BCG. All mice with BCG were tested for tuberculin skin test (TST) so as to determine susceptibility and resistance against tuberculosis.

All the three groups were challenged with (0.5ml)virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, ATCC 35718) *M. tuberculosis*.10^7(CFU) .

Another group of mice n=6 for the study of humoral response by immunization of mice with immune serum and challenged with *M. tuberculosis* Following by two groups of mice n=10 for each group A and B for the susceptibility and resistance of the strains of mice by immunization of mice with BCG for 21 days and testing by tuberculin skin test (TST). The efficacy was based on a survival rate of challenged mice, mortality rate and bacterial load of *M. tuberculosis* in the lungs of infected mice.

Results: After three weeks of observations Tuberculin skin test reaction for the BCG immunized mice were positive, hence the mice strain of BALB/c were susceptible and Swiss white mice were resistant for BCG.

Survival mice in group (A) were 50%, group (B) 70% and group(C) 0%.The mortality rates for (A) 50%, (B) 30% and (C) 100%. The immunopotency and protective efficacy of BCG first dose and boosting dose were (50%) and (70%) respectively. Humoral immunity response against *M. tuberculosis* showed negative reaction hence mortality rate was 100%.

Conclusions: The incidence of TB is high inspite of primary vaccination in neonatal period and therefore requires consideration for repeated immunization of BCG.

Keywords: BCG**,** *M. tuberculosis***,** BALB/c Mice, Swiss white mice, tuberculin skin test (TST).

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Introduction

Tuberculosis (TB)is primarily a chronic lung infection that is one of the most potent and wide-spread human infections today, and a major cause of death from bacterial pathogens (Lawn, and Zumla;2011).). It affects more young adults and therefore has a high impact on the socioeconomic status of people (Zakham *et al.*, 2012).In Africa, investigations of TB is complicated by the parallel epidemic of HIV because co-infection is common. This makes it necessary to consider HIV infection, especially in high HIV prevalent areas (Morris *et al.*, 2011).) in 2015 an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children.(WHO, 2016).

The BCG vaccine can provide effective protection against TB in up to 8 out of 10 people who are given it. Currently, BCG vaccinations are only recommended for groups of people who are at a higher risk of developing TB. in this context, *M. bovis* BCG is the only vaccine available for the prevention of tuberculosis in humans. The live, attenuated BCG vaccine, originally derived by serial passage of a virulent strain of *M. bovis*, has been used to prevent tuberculosis since 1921. The BCG is effective against severe forms of childhood tuberculosis but appears to be of limited efficacy against adult pulmonary disease in endemic areas. (Schreiber, *et al*; 2010).

BCG vaccination includes children living in areas with high rates of TB, or those who have close family members from countries with high TB rates, and people under the age of 16 who are going to live and work with local people in an area with high rates of TB for more than three months. It's also recommended that some people, such as health care workers, are vaccinated because of the increased risk of contracting TB while working.(Schreiber, *et al*; 2010).

Mycobacterium bovis based Bacille Calmette Guerin (BCG) was originally used as an oral vaccine in the 1930s.The movement from oral administration to intradermal injection began in the 1960s. *M. Bovis* originally infects the gastrointestinal tract of cattle and humans naturally. The BCG based vaccine can provide stimulation of both innate and acquired immunity.(Schreiber, *et al*; 2010).

Despite early success, the BCG vaccine has had a limited effect against the incidence of TB in the developing world. Various clinical trials have demonstrated that BCG showed variable levels of efficacy against pulmonary TB. For example, a major trial in the United Kingdom showed >75% protection(Dietrich, *et al*;2003). However, trials in south India and Malawi

demonstrated that BCG failed to protect consistently against pulmonary (Ferraz, *et al*;2004) . The reasons for this have been a matter of debate and this indicates an urgent need for more effective vaccines to decrease the incidence of tuberculosis.(Dietrich, *et al*;2003).

Materials and methods

A total number of 56 mice were examined for their immunopotency and protective efficacy of BCG against challenge dose of *M. tuberculosis* as single and second repeated dose. In experiment contains three groups of inbred BALB/c male mice of 6-8 weeks of age, weighing 25-30 grams mice each of ten mice n = 10 (30mice). The first group(A) were immunized with (0.5ml) of BCG which diluted with 10ml of normal saline in sterile container in ice for 21days as first dose (I.P) for 21 days. The second group (B) were immunized with BCG (I.P) as first dose for 15 days and boosting dose (second dose) for another 15 days. The third group(C) were not immunized with BCG. All mice with BCG were tested for tuberculin skin test (TST) so as to determine susceptibility and resistance against tuberculosis.

Tuberculin skin Test procedure:

Intradermal injection of mice with 0.2 ml of purified protein derivative (PPD) and then induration and swelling were observed in the skin of tested mice after 24-72 hours.

All the three groups were challenged with (0.5ml)virulent *M. tuberculosis* H37Rv strain (American Type Culture Collection, ATCC 35718) *M. tuberculosis*.10^7(CFU) .

Challenge dose:

Preparation of different concentration of *M. tuberculosis* by McFarland 0.5 and colony forming unit (CFU) of 10^2 ,10^3,10^4, upto 10^9.

rrMcFarland standards were made by mixing specified amounts of Barium chloride and Sulfuric acid together. Mixing the two compounds forms a Barium Sulfate precipitate, which causes turbidity in the solution. A 0.5 McFarland standard is prepared by mixing 0.05 ml of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), with 9.95 ml of 1% sulfuric acid (H_2SO_4).The standard was compared visually to a suspension of bacteria in sterile saline or nutrient broth. If the bacterial suspension was too turbid, it was then diluted with more saline. If the suspension was not

turbid enough, more bacteria was added.

Preparation of inoculum for LD_{50} determination of M.tb.

On the day of inoculation the optical density($O.D._{540nm}$) of bacterial suspension of *M. tuberculosis* was adjusted to 1.35 and 1ml of suspension then serial dilutions (10^0 ,10^1 ,10^2,10^3,10^4, up to 10^9) were prepared in saline, for each dilution 6 mice of each group were inoculated with 0.5ml intrapritoneally.Inoculation of 0.5 ml of different concentrations 10^2,10^3,10^4, up to 10^9 of *M. tuberculosis*. McFarland 0.5 intrapritoneally in a group of 6 mice for each concentration to determine the LD_{50}.The concentration of the suspensions which kills 50% of the mice is the LD_{50}.

The control group received normal saline 0.5ml intrpritoneally. Observations of the two groups were done for one week after the inoculation of different concentrations of *M. tuberculosis* and the normal saline were recorded. Lethal dose (LD_{50}) concentration which kills 50% of the mice was recorded .

Another group of mice n=6 for the study of humoral response by immunization of mice with immune serum and challenged with *M. tuberculosis* Following by two groups of mice n=10 for each group A and B for the susceptibility and resistance of the strains of mice by immunization of mice with BCG for 21 days and testing by tuberculin skin test (TST). The efficacy was based on a survival rate of challenged mice, mortality rate and bacterial load of *M. tuberculosis* in the lungs of infected mice.

Preparation of mice tissues for histopathology

For histological study, the lungs from four dead mice after receiving BCG immunization for 21 days and then challenged by *M. tuberculosis* for 3 weeks later, and then were fixed by absolute ethanol, embedded in paraffin, sectioned and stained with haematoxylin and eosin (Hernandez-Pando, et al;1996) In these slides the area of granuloma and the percentage of lung area affected by pneumonia were determined.

Results

Results of tuberculin skin test in BCG imunized mice

A total number of 10 inbred BALB/c mice were tuberculin skin test (TST) positive after immunization with BCG while out bred Swiss white mice were negative for TST.

Table (1) The results of tuberculin skin test reaction (TST)

Type of mouse strain	No. of mice/group	TST reaction
BALB/c inbred susceptible mouse	10	Positive
Swiss out bred resistant mouse	10	Negative

Results of LD_{50} concentration:

Figure (1)shows the groups miceinoculatedwith different concentrations of *M.tuberculosis*. (10^2,10^3,10^4, up to 10^9) and the normal saline(control group) for one week showed thatthegroup of colony forming unit (CFU)(10^0) exhibited no death of mice.Mice given (10^1) and(10^2) were exhibited 2deaths out of 10 (2/10) i.e mortality ratio 20% The groups of (10^3) and (10^4)showed 3 deaths out 10 (3/10)i.e mortality ratio 30%.In the groups given(10^5) and(10^6) 4deaths out 10 (4/10) mice showed mortality ratio 40% .showedCFU (10^7) of mice showed 5deaths out of 10 (5/10)with mortality ratio 50%. This showed that the lethal dose (LD_{50}) which killed 50% of the total number of the mice.

Table (2)Shows the results of LD_{50} determination

No. of BALB/c mice /group	**Bacterial inoculum(CFU)**	**No. of dead mice**	**Mortality ratio**
10	**10^0**	**0**	**0%**
10	**10^1**	**2**	**20%**
10	**10^2**	**2**	**20%**
10	**10^3**	**3**	**30%**
10	**10^4**	**3**	**30%**
10	**10^5**	**4**	**40%**
10	**10^6**	**4**	**40%**
10	**10^7**	**5**	**50%**
10	**10^8**	**6**	**60%**
10	**10^9**	**7**	**70%**

Results of vaccination with single and repeated (booster dose) of BCG in mice

Survival mice in group (A) were 50%, group (B) 70% and group(C) 0%.The mortality rates for (A) 50%, (B) 30% and (C) 100%. The immunopotency and protective efficacy of BCG first dose and boosting dose were (50%) and (70%) respectively, and mortality rates of BCG first dose and boosting dose were (50%) and(30%) respectively .For Group(C) which were not immunized 100% mortality and 0% protective efficacy after challenge dose of *M. tuberculosis*.

Table (3) The results of single and repeated (booster doses) of BCG vaccination in mice

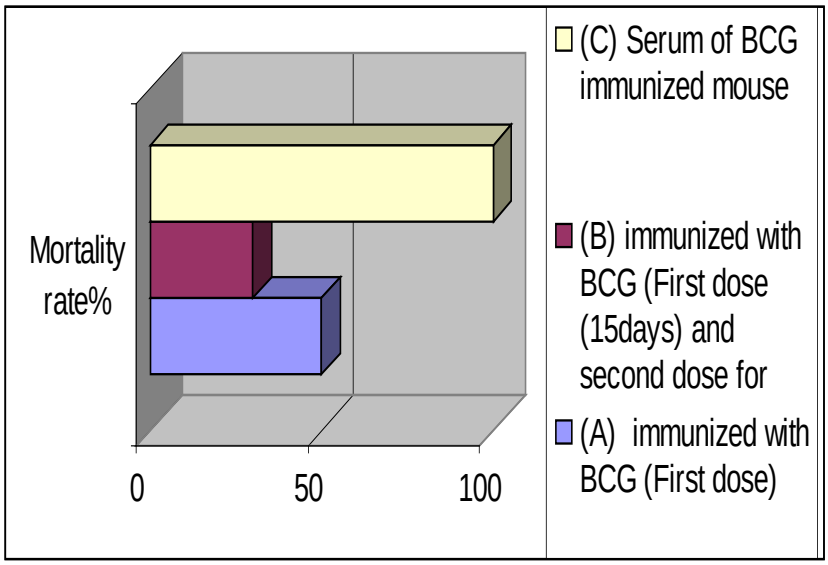
Group of mice	Number of mice/group	No.of survival mice after challenge dose of M.tb.	No.of dead mice	Protective efficacy%	Mortality rate%
(A) immunized with BCG (First dose)	10	**5**	**5**	**50%**	**50%**
(B) immunized with BCG (First dose (15days) and repeated dose for 15days)	10	**7**	**3**	**70%**	**30%**
(C)only challenge dose of M.tb.)	10	**0**	**10**	**0%**	**100%**

The results of Humoral response of mice against *Mycobacterium tuberculosis*.

The result was death of the mice within 24hours, and mortality rateof humoral response of mice against *Mycobacterium tuberculosis* was 100% compared with 50% BCG first dose ,30% BCG boostingdose and 16.7% mortality rate of transfer factor (TF) .(Fig.8,9).

Table (4) The results of Humoral response of mice against *Mycobacterium tuberculosis*.

Group of mice	Mortality rate%
(A) immunized with BCG (First dose)	50
(B) immunized with BCG (First dose (15days) and second dose for 15days)	30
(C) Serum of BCG immunized mouse	100



Figure(1)Shows Humoral response of mice against *Mycobacterium tuberculosis.*

Results of lung histopathology of mice

All slides of dead mice stained with haematoxylin and eosin showed lung bacilli that Was *M. tuberculosis*.

Discussion

In this current study a single dose of BCG vaccination intrpritoneally (i.p) was used in BALB/c mice which were free of pathogen for a period of 21days to evaluate the efficacy of BCG and the effect of repeat dose of MTB-H37Rv. Vaccination measured by Delayed type hypersensitivity (DTH), survival of challenged mice and bacterial load in the infected lungs of mice. In this current study repeated dose (boosting dose) of BCG increased the survival of challenged mice which were found that the survival rate was 70% comparing with 50% survival rate of BCG single dose. The results obtained is in accordance with a study done in India by Kashyap 2010 (Assessment of immune response to repeat stimulation with BCG vaccine using *in vitro).* Their data showed that induction of repeat dose of BCG in the peripheral blood mononuclear cells (PBMC) model increased specific antimycobacterial immune responses (anti-BCG IgG and IFN-γ, T cell activity-ADA). In other words they had shown that culturing of human PBMC's with repeat dose of BCG showed increased memory response to previous immunization(Kashyap, *et al;*2010) Recently P M Udani(1989) has raised a question of whether repeat doses are needed in countries where the burden of TB is high .They suggestsed that the efficacy of BCG may improve with repeat doses. Secondly, these results may be helpful in designing future experiments in animal models with respect to a booster approach .(Uyan *et al* ;2000)Ultimately, with the help of all experimental evidences, they conclude that India may start repeat immunizations of BCG and may reduce the burden of TB in future generation. There are some countries that give repeated doses of BCG vaccine. For example, in Turkey BCG immunization is done four times: during infancy, at two months after birth, at six to seven years of age (first grade), at eleven to twelve years of age (fifth grade), and sixteen to seventeen(16-17) years of age (high school)(Ponnighaus,*et al*;1992).

In this current study confirmed that BALB/c mice has a positive tuberculin skin test response compared to outbred Swiss mice, indicating that the delayed-type hypersensitivity (DTH) to *M. tuberculosis* antigens is diminished. In this current study BCG vaccination measured through survival of challenged mice, DTH and bacterial load in the lungs of infected mice. The efficacy of BCG against challenge dose of *M. tuberculosis* was 50% and mortality rate was 50% The obtained results are similar to previous studies done by Turner; *et a*l in 2001.Who demonstrated used low-dose aerosol infection of *M. tuberculosis* to compare chronic tuberculosis.

Susceptible mice, which are often able to contain bacterial growth in the liver and spleen, are unable to restrict growth in the lung. While granulomas in resistant mice are well organized, consisting of aggregated lymphocytes and macrophages, lesions in susceptible mice are often poorly organized, necrotic and contain few lymphocytes. This implies that susceptible strains have a defect in recruiting or retaining lymphocytes in the lung. The production of cytokines crucial for the control of tuberculosis ,such as IFNg, is usually diminished in susceptible mice, resulting in a general delay in the effect or phase of the adaptive immune response. In many cases, susceptible mice are deficient in maintaining a single dose.(Flynn,1995).

Conclusion

Inbred strains of mice exhibit varied patterns of susceptibility following infection with virulent *M tuberculosis*. Susceptible mice have progressive fulminate disease resulting in their premature death; in contrast, resistant mice are able to control bacterial replication, limit lung injury and survive longer.

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