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Research Article

Evaluation of the Immunopathological Response to BCG Vaccine in a Xenogeneic Immunocompetent Animal

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Abstract: *Mycobacterium bovis,* the causative agent of bovine tuberculosis (BTB), is one of the most significant endemic diseases confronting government, veterinary professionals, and farming industry worldwide nowadays. *M. bovis* has not only a negative impact on bovine health and economy, but also poses a threat to public health as a zoonotic disease that could be transmitted from animal to human. Although, bacillus Calmette-Guérin (BCG) vaccine of *M. bovis* has been extensively used in many animals, only few studies had reported its side effects in these animals. In this study, the systemic pathological lesions and immunoglobulin levels associated with intranasal (IN) and subcutaneous (SC) injection of BCG vaccine in Swiss male mice have been evaluated. The results revealed an elevation in IgM and IgA levels in both routes (nebulization, subcutaneous injection) while there was a dramatic increase in IgG levels in subcutaneously injected mice. Aerosolization of BCG vaccine using a nebulizer resulted in severe pulmonary lesions with numerous megakaryocytes in the spleens of mice. On the other hand, SC injection had mild effect on pulmonary tissues and induced moderate extramedullary hematopoiesis in the hepatic tissues of mice. In conclusion, inadvertent vaccination of BCG in Swiss mice, triggered adverse tissue reaction and remarkable increase in Ig level. The severity of tissue lesions corresponded to the injection route in mice.

Keywords: *Aerosol, BCG, Extramedullary hematopoiesis, Mice, Vaccine*

Ksenojenik İmmünokompetan Bir Hayvanda BCG Aşısına İmmünopatolojik Yanıtın Değerlendirilmesi

Öz: Sığır tüberkülozunun (BTB) etkeni olan *Mycobacterium bovis*, günümüzde dünya genelinde hükümetlerin, veteriner hekimlerin ve tarım endüstrisinin karşı karşıya kaldığı en önemli endemik hastalıklardan birisidir. *M. bovis* sadece sığır sağlığı ve ekonomisi üzerinde olumsuz bir etkiye sahip olmakla kalmayıp, aynı zamanda hayvandan insana bulaşabilen zoonotik bir hastalık olarak halk sağlığı için de tehdit oluşturmaktadır. *M. bovis* basilinin Calmette-Guérin (BCG) aşısı birçok hayvanda yaygın olarak kullanılmasına rağmen, sadece birkaç çalışma bu hayvanlarda yan etkilerini bildirmiştir. Bu çalışmada, İsviçre erkek farelerinde BCG aşısının inhalasyon ve subkutan (SC) enjeksiyonu ile ilişkili sistemik patolojik lezyonlar ve immünoglobulin seviyeleri değerlendirilmiştir. Sonuçlar, her iki yolla da (nebülizasyon, subkutan enjeksiyon) IgM ve IgA seviyelerinde artış olduğunu ortaya koyarken, subkutan olarak enjekte edilen farelerde IgG seviyelerinde dramatik bir artış olduğunu göstermiştir. BCG aşısının bir nebülizör kullanılarak aerosolize edilmesi, farelerin dalaklarında çok sayıda megakaryosit içeren ciddi pulmoner lezyonlarla sonuçlanmıştır. Öte yandan, SC enjeksiyonunun pulmoner dokular üzerinde hafif bir etkisi olmuş ve farelerin hepatik dokularında orta derecede ekstramedüller hematopoezi indüklemiştir. Sonuç olarak, İsviçre farelerinin BCG ile yanlışlıkla aşılanması, olumsuz doku reaksiyonunu ve Ig seviyesinde kayda değer bir artışı tetiklemiştir. Doku lezyonlarının şiddeti, farelerdeki enjeksiyon yolu ile uyumluluk göstermiştir.

Anahtar sözcükler: *Aerosol, BCG, Ekstramedüller hematopoez, Fare, Aşı*

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Introduction

Mycobacterium bovis is a member of *Mycobacterium tuberculosis* complex (MTBC), the main causes of tuberculosis (TB) in livestock and wildlife across the globe and most noticeably in Africa and Asia [1-6]. Bovine tuberculosis (BTB) is a zoonotic disease that not only has a substantial impact on the world economy and animal health, but also poses a hazard to both animals and humans [7]. It has been estimated that cases of BTB among cattle worldwide will be greater than 50 million annually with $3 billion loss in economy [8]. Therefore, there is an urgent need for BTB control strategies, especially in low- and middle-income countries and other regions where test-and-slaughter methods are not feasible and standardized [9]. MTBC are known to induce granulomatous-caseous-necrotizing lesions mainly in the lungs and regional lymph nodes, but they could also induce lesions in the liver, spleen, kidneys, mammary glands, pericardium, uterus, and brain [10].

In Egypt, BTB is considered one of the most significant animal health hazards since cattle represent the main source of meat and milk in Egyptian community and their health status have a great impact on economy and social life [11]. In the recent years, there was an increase in the incidence of BTB due to the export of live animals from endemic regions with a high prevalence of *M. bovis* [12, 13].

The most effective method of controlling BTB is known to be vaccination combined with reliable diagnostic testing. Currently, the only alternative for protecting humans and livestock against tuberculosis is the live attenuated Bacillus Calmette-Guérin (BCG) vaccine [14]. BCG was originally intended as a cattle vaccine, and its efficacy against BTB is still debated [4]. However, modest clinical symptoms have been observed on rare occasions during BCG vaccine testing in a few animal species [15]. The individual's age, immunological status, vaccination dose, strain, and route of administration of BCG vaccine are the main variables that could predict the severity of tissue reaction to BCG vaccine [16]. Most tissue reactions against BCG were reported to be local/regional and self-limiting, whereas suppurative lymphadenitis and abscessations were the most severe tissue reactions that occurred in some cases [15]. A large dose of BCG administered subcutaneously to cattle resulted in topical lesions with no further problems, and the TB bacilli were cleared from the body, but following oral administration of BCG to mice, mild side effects were observed including infrequent cervical lymphadenitis [17, 18]. Although large doses of intranasal BCG provided better lung protection, it also caused BCG postvaccinal granulomatous pneumonia [19]. Moreover, few studies focused on the parenchymatous lesions and altered Ig levels caused by live attenuated BCG vaccine.

Consequently, the aim here is to study the histopathological alterations in parenchymatous organs after intranasal aerosolization (IN, via nebulization) and subcutaneous injection (SC) of BCG vaccine in xenogeneic Swiss mice and evaluate the changing in Ig level of vaccine mice.

Materials and Methods

Ethical Statement

The Benha University ethical committee for animal experiments approved all procedures that involved the handling and collection of blood samples (approval Nr. BUFVTM 02-09-22).

Vaccine

Servac Freeze-dried BCG vaccine used in trials of the present study, was prepared from a living attenuated BCG and *M. bovis* strain. It was produced by the Veterinary Serum and Research Institute (VSRI, Abbasia, Egypt).

Experimental Animals

For this study, 15 Swiss male mice with one and half month age, weighing 20 g were purchased from the Center for Laboratory Animals at Benha University's Faculty of Veterinary Medicine in Egypt. Before the experiment, all mice were acclimated for two weeks (in a light/dark cycle of 25±2°C and 12:12 h) and given a standardized pellet meal and free access to water.

Vaccination Protocol

As stated in *Table 1*, the 15 mice were divided into three groups, each with five mice. Group 1 (control) mice were not immunized. Mice in groups 2 received 0.1 mL Servac BCG vaccine by SC injection, while mice in group 3 received 0.1 mL from the vaccine via IN using a nebulizer (Uhde GmbH, Germany) for 5 min. After 21 days, all of the mice in the three groups were scarified.

Blood Sampling and Serological Analysis

Before the mice were euthanized, they received an intraperitoneal (IP) dosage of 120 mg/kg of ketamine (100 mg/mL) to anaesthetize them, then blood samples were obtained from the retro-orbital plexus in collecting tubes containing dipotassium ethylenediamine tetra acetic acid. Serum was separated from the collected blood samples after centrifugation of the blood tubes for 15 min at

Table 1. *Animal groups and vaccines used in the study*

Group	Animals	Route and Dose
1 (control)	Five mice	Non-vaccinated
2 (SC- vaccinated mice)	Five mice	0.1 mL SC and booster dose 14 days later
3 (IN- vaccinated mice)	Five mice	0.1 mL IN and booster dose 14 days later

1200xg. Immunoglobulins (Ig) IgM, IgA, and IgG levels were assessed. Each Ig was tested using enzyme-linked immunosorbent assays (ELISAs): rat IgG, IgA, and IgM ELISA kits (Creative Biolabs, USA).

Histopathological Analysis

After 21 days of vaccination, all mice were euthanized and tiny samples of lung, spleen, kidney, liver, and lymph node tissue were taken from each mice. The specimens were fixed for 72 h in 10% neutral-buffered formalin, dehydrated, cleaned, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) dye. Using a Nikon eclipse E800 microscope with an OMAX eye-piece camera, photomicrographs of histopathological changes were taken.

Statistical Analysis

The data was analyzed using the Graph Pad Prism 6.0 software (San Diego, USA). Parametric one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons tests were used to compare outcome variables between groups.

Results

Serum Immunoglobulin Levels

The BCG vaccine induced remarkable changes in the level of immunoglobulins in xenogeneic mice via both routes. In comparison to control unvaccinated mice, there was a significant rise in the level of IgG in the sera of both IN (P=0.0015) and SC (P<0.0001) vaccinated mice *(Fig. 1-A; Table 2)*. Notably, mice with the SC immunization of BCG had much higher IgG levels than mice with the IN administration. The level of IgM and IgA in the blood of both IN and SC vaccinated mice was also significantly higher than that in the unvaccinated mice (P<0.0001) *(Fig. 1-B,C; Table 2)*.

Histopathology

In most of the examined parenchymatous organs, the control group exhibited no degenerative alterations or inflammation. In contrast, mice vaccinated via both routes had varying degrees of cell damage and inflammation. There were no significant microscopic lesions in the lungs of control mice *(Fig. 2-A)*.

The IN-vaccinated mice had more severe pulmonary lesions than the SC-vaccinated mice. Bronchioles had significant hyperplasia/hypertrophy of the epithelial lining, as well as intraluminal homogeneous eosinophilic material infiltrated with cellular debris *(Fig. 2-B)*. The majority of the mice in this group had significant peribronchial mononuclear cellular infiltration. Congestion of interalveolar blood vessels was the frequent finding in all the mice. There was also multifocal interalveolar inflammatory cellular aggregations specially lymphocytes in many examined lungs *(Fig. 2-C)*. Intra alveolar oedema and hemorrhages were reported in 75% of the mice *(Fig. 2-D)*. Hyperplasia of pneumocytes type II was also prominent in all the lungs where the hemorrhage was extensive *(Fig. 2-D)*.

In SC vaccinated mice, BCG had less adverse effects compared to IN vaccinated mice. Mild bronchial mucosal hyperplasia was evident in many mice in SC vaccinated

Table 2. *The means and standard deviations of immunoglobulin concentrations in control, vaccinated mice by BCG vaccine*

Immunoglobulin	Control		IN-vaccinated Mice		SC-vaccinated Mice	
	Mean	SD	Mean	SD	Mean	SD
IgG	390.8	64.3	755.2	50.9	1710.8	209.3
IgM	215.0	26.7	584.2	26.7	779.8	86.5
IgA	357.8	48.4	606.4	19.6	700.4	32.3

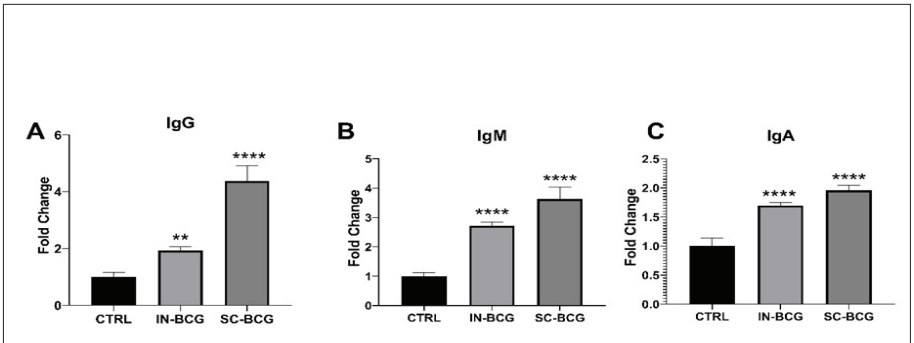

Fig 1. Serum levels of immunoglobulins (IgG, IgA and IgM) in vaccinated and unvaccinated mice. The graph represent the fold increase in Ig level following vaccination compared to unvaccinated mice. Significance difference is indicated as ****P≤0.0001, **P≤0.01

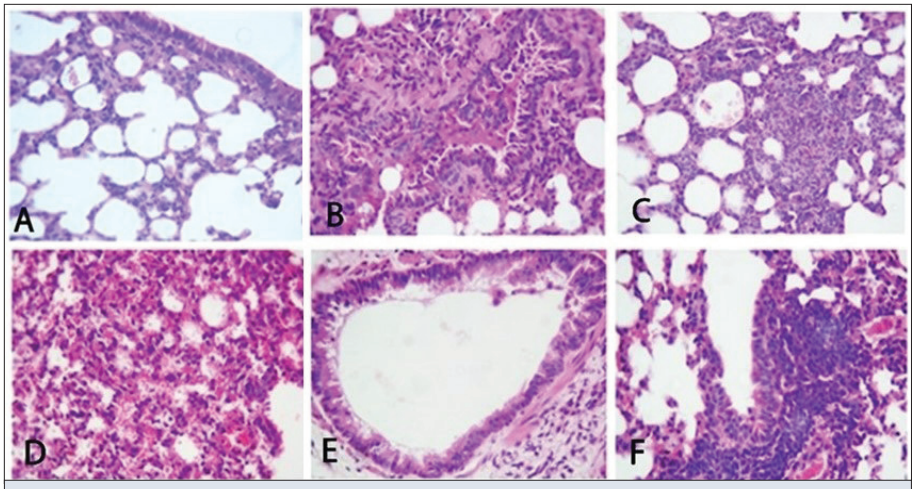

Fig 2. Pulmonary photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** lung of control group showing nearly normal alveoli and interstitial tissue. **B-D)** Lungs of IN vaccinated mice showing hyperplasia/hypertrophy of the epithelial lining with intraluminal homogeneous eosinophilic material (B), interalveolar inflammatory cellular aggregations (C), and Intra alveolar oedema and hemorrhages (D). **E-F)** Lungs of SC vaccinated mice showing mild bronchial mucosal hyperplasia (E), and foci of peribronchial mononuclear inflammatory cellular infiltration (F)

mice *(Fig. 2-E)*. Foci of peribronchial mononuclear inflammatory cellular infiltration were also seen in few mice in this group *(Fig. 2-F)*.

The control group's livers showed no significant microscopic alterations *(Fig. 3-A)*. The adverse effect of BCG vaccination was more pronounced in the mice vaccinated via IN compared to SC route. The hepatic parenchyma of IN-vaccinated mice showed foci of extramedullary haematopoiesis (EH) and Kupffer cell infiltrations. Many of the examined livers in this group had hepatocellular deterioration in the form of hydropic degeneration *(Fig. 3-B)*. In many mice, there were many hepatocytes with basophilic cytoplasm in the hepatic parenchyma *(Fig. 3-C)*. Some mice in this group had multifocal areas of hepatic necrosis mixed in with inflammatory cells (mostly mononuclear and a few giant cells) *(Fig. 3-D)*. Congestion of hepatic sinusoids with multifocal areas of hemorrhage were also observed in IN-vaccinated mice *(Fig. 3-E)*. Thrombosis of some central veins was among the incidental findings in few mice. Furthermore, periductal moderate infiltration of mononuclear cells was detected in a few IN-vaccinated mice *(Fig. 3-F)*. In SC vaccinated mice, mild hepatocellular degeneration with few foci of hepatic necrosis were evident in some examined livers. Many mice had central vein congestion with perivascular focal mononuclear cellular aggregations *(Fig. 3-G)*. Mild hyperplasia of the bile ducts with periductal fibrosis and inflammatory cellular aggregation were seen *(Fig. 3-H)*.

The control mice had no noticeable kidney lesions,

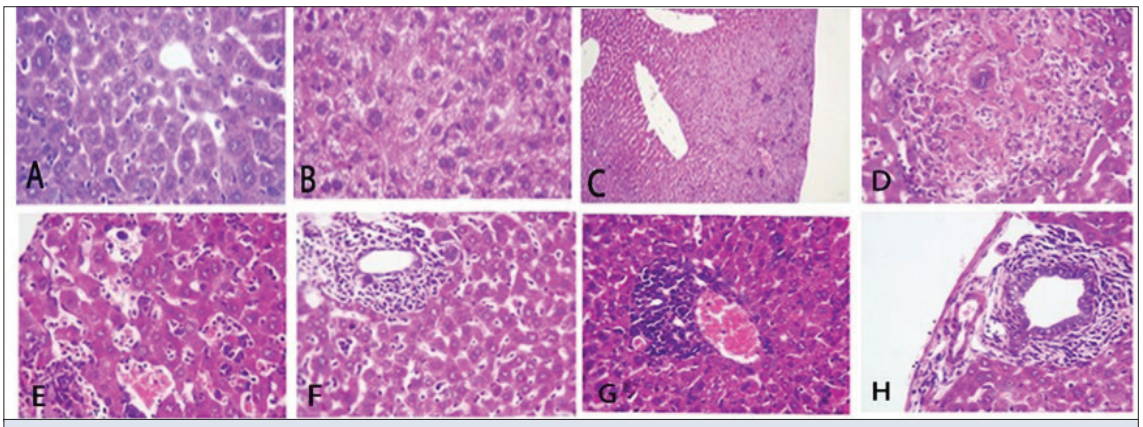

Fig 3. Liver photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Liver of control group showing normal hepatocyte and patent sinusoids. **B-F)** Liver of I/N vaccinated mice showing hydropic degeneration (B), hepatocytes with basophilic cytoplasm in the hepatic parenchyma (C), multifocal areas of hepatic necrosis mixed in with inflammatory cells (D), congestion of hepatic sinusoids with multifocal areas of hemorrhage (E), and moderate periductal mononuclear cells infiltration (F). **G-H)** Liver of SC vaccinated mice showing central vein congestion with perivascular focal mononuclear cellular aggregations (G), and mild hyperplasia of the bile ducts with periductal inflammatory cellular aggregation (H)

whereas both vaccinated groups had similar renal lesions *(Fig. 4-A)*. Proliferative glomerulonephropathy was the most common microscopic lesion in the kidneys of IN-vaccinated mice *(Fig. 4-B)*. In IN-vaccinated mice, necrotic cellular debris was seen in the bowman's space of some glomeruli *(Fig. 4-C)*. Periglomerular and intertubular hemorrhage were noted in some mice in this group *(Fig. 4-C)*. The IN-vaccinated mice showed a lot of basophilic cytoplasm in their renal tubular epithelia *(Fig. 4-D,E)*. In SC-vaccinated mice, there were a few foci of infiltrating intertubular and periglomerular mononuclear inflammatory cells in the kidney *(Fig. 4-F)*.

Control mice showed no significant microscopic changes in their heart *(Fig. 5-A)*. Degeneration (vacuolation) of cardiomyocytes was prominent in IN-vaccinated mice compared to SC-vaccinated mice *(Fig. 5-B)*. Congestion and oedema of intermuscular blood vessels were evident in IN-vaccinated mice *(Fig. 5-C)*. Cardiomyocytes with basophilic cytoplasm and marked loss of striations were evident in many IN-vaccinated mice *(Fig. 5-D)*. On the other hand, no degeneration was detected in the cardiomyocytes in SC-vaccinated mice. Similar to IN-vaccinated mice, cardiac muscles with basophilic cytoplasm were also seen in some mice in this group.

No significant microscopic alterations were seen in the spleens of the control mice *(Fig. 5-E)*. Megakaryocyte

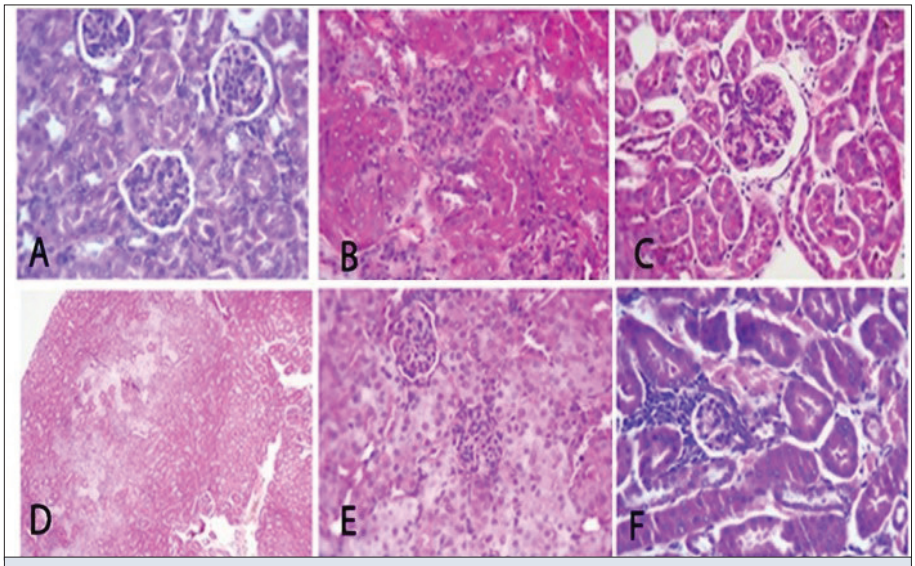

Fig 4. Kidney photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Kidney of control group showing normal renal glomeruli as well as normal renal tubules. **B-E)** Kidney of IN vaccinated mice showing proliferative glomerulonephropathy (B), necrotic cellular debris in the bowman's space (C), and basophilic cytoplasm in the renal tubular epithelia (D-E). **F)** kidney of SC vaccinated mice showing intertubular and periglomerular mononuclear inflammatory cells

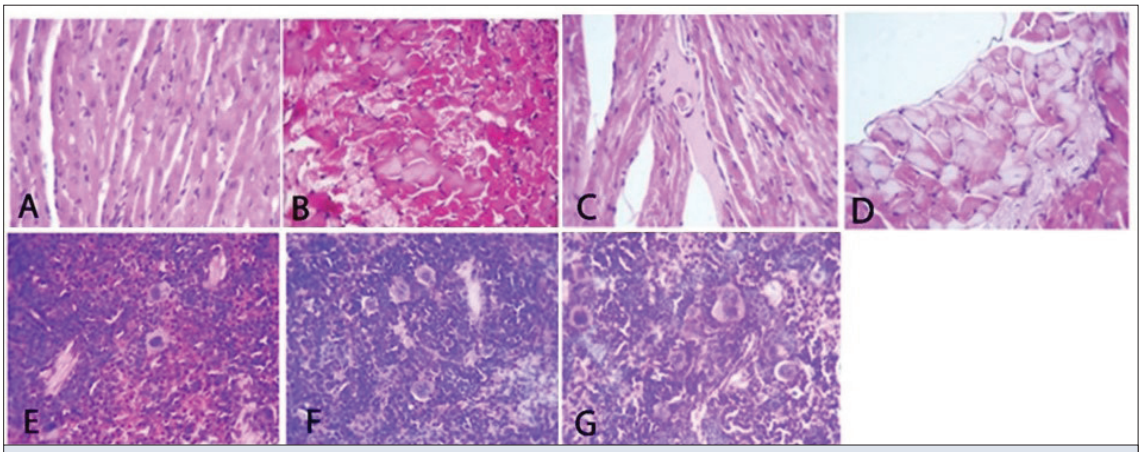

Fig 5. Heart and spleen photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Heart of control group showing cardiomyocyte. **B-D)** Heart of IN vaccinated mice showing vacuolation of cardiomyocytes (B), congestion and oedema of intermuscular blood vessels (C), and basophilic cytoplasm and marked loss of cardiomyocytes striations (D). **E)** Spleen of control group showing normal microscopic structure. **F)** Spleen of IN vaccinated mice showing low number of megakaryocyte proliferation. **G)** Spleen of SC vaccinated mice showing high number of megakaryocyte proliferation

proliferation was evident in both IN and SC-vaccinated mice, but it was more significant in SC-vaccinated mice than in IN-vaccinated mice *(Fig. 5-F,G)*.

Discussion

BCG is one of the most popular and safest human vaccine against tuberculosis worldwide [14]. Although BCG has 70-80% effectiveness against the most serious complications of TB, i.e TB meningitis, it is less potent in preventing pulmonary tuberculosis [20]. Reports of adverse reactions to BCG are relatively rare in healthy immunocompetent individuals while serious adverse reactions were recorded in some immuno-compromised individuals [21]. Many factors affect the development of various adverse reactions to BCG vaccine including the potency and dose of the vaccine strain, administration route, age, and host immunity [21]. Post vaccinal adverse reactions and complications in humans are mainly in the form of mild and transient fever, injection site abscesses, lymphadenitis, skin rash, and systemic disseminated BCG infection [22]. Although BCG has also been widely used in vaccine research in laboratory animal hosts, and it is currently being developed for usage in a range of livestock and wild animals, the detrimental effects of BCG on lab animals i.e mice, however, are not well understood [23]. In this study, we studied the adverse effect of BCG vaccine in xenogeneic mice using two different routes (IN and SC) and assessed the alteration in the immunoglobulin levels post vaccination.

The results confirmed that BCG vaccine significantly increased the level of IgG in the sera of both IN and SC vaccinated mice and caused notable alterations in the level of immunoglobulins in xenogeneic mice via both routes. Additionally, SC immunization caused a greater rise in all immunoglobulins than nebulized BCG vaccination and compared to control mice.

The obtained results come in accordance with previous findings of Husain, Kashyap [24] and Husain, Warke [25] where they observed increased IgG level in comparison with IgA and IgM in mice after BCG vaccination. In addition, Medeiros, Armôa [26] found significant increase in IgG1 following vaccination of BALB/c mice with BCG vaccine. Contrary to these results, other studies has found that intranasal BCG vaccination significantly increases IgA upregulation compared to subcutaneous BCG vaccination and unvaccinated mice [27]. Mice are thought to be more resistant to *M. tuberculosis* infection than humans, and they could tolerate large numbers of *Mycobacterium* in their lungs for months without apparent progression of disease [28].

Our results suggest that BCG vaccination have adverse effects on parenchymatous organs which are route-dependent. The severity of the effects was more pronounced in mice vaccinated via the IN route, implying that aerosolization of BCG induces an inflammatory response with moderate irritation of the respiratory tract. The hepatic lesions demonstrate that BCG vaccination using IN route alters hepatic architecture and promotes more extramedullary hematopoiesis and Kupffer cell infiltrations compared to the SC route in rats.

The present findings showed that BCG vaccine induced moderate granulomatous response in the lungs of mice after 21 days of vaccination without showing intragranulomatous necrosis. This finding has been observed in some of previous studies [29]. As an argument for this, mice immunized with mucosal vaccination had a higher lymphocyte proliferation than mice immunized with SC route [30].

Consistent with our findings, intraperitoneal BCG vaccination induced significant inflammatory cellular infiltration and hepatocyte damage in 3 weeks post vaccination [31]. Moreover, it has been reported that BCG induced granulomas in some organs, mainly lungs and liver, though these granulomas may represent a natural immune response [32].

Megakaryocyte proliferation and extramedullary hematopoiesis in spleen and livers were prominent in all BCG vaccinated mice in this study. Similarly, other studies have reported these findings in the mice post vaccination [33,34]. In addition, platelets and the megakaryocyte of which they were developed, perform a range of immune functions including activating and adhering leukocytes and endothelium, creating extracellular traps for neutrophils, sensing pathogens, and clearing them [35,36]. As part of an immune response to pathogens, extramedullary hematopoiesis occurs as well. This response is mainly directed towards the spleen and liver, where antigen-presenting cells and phagocytes take place [35].

Both vaccinated groups endured comparable renal lesions. However, cardiac degeneration was one of the most severe lesions observed in IN vaccinated mice compared to SC vaccinated mice. BCG can be phagocytosed and degraded by macrophages, giving rise to various immunogenic components [37] that can strongly stimulate an inflammatory response through the activation of different pattern recognition receptors (PRRs) [38].

The limitation of the current study was the vaccine should be evaluated with more routes and study the immunological and pathological effects of these routes.

The present findings clearly showed that a change in the route of administration of BCG to mimic the natural route of infection might be a successful strategy to prevent pulmonary tuberculosis. However, further researches are needed to avert aggravating undesirable concerns.

Availability of Data and Materials

The datasets analyzed during the current study are available from

the corresponding author (A. Selim) on reasonable request.

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Conflict of Interest Competing Interests

The authors declare that they have no conflicts of interest.

Author Contributions

Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft preparation, S.A.S., A.S., M.M.G., S.M.E., M.H.W and M.A.M.; writing-review and editing, S.A.S., A.S., M.M.G., M.H.W., S.M.E. and M.A.M.; project administration, S.A.S., A.S., M.M.G., S.M.E. and M.A.M. All authors have read and agreed to the published version of the manuscript.

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