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Avoiding COVID-19 complications with diabetic patients could be achieved by multi-dose Bacillus Calmette–Guérin vaccine: A case study of beta cells regeneration by serendipity

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Abstract:

Diabetes mellitus (DM) is one of the major risk factors for COVID-19 complications as it is one of the chronic immune-compromising conditions especially if patients have uncontrolled diabetes, poor HbA1c &/or irregular blood glucose levels. Diabetic patient's mortality rates with COVID-19 are higher than cardiovascular or cancer patients. Recently Bacillus Calmette–Guérin (BCG) has shown successful results in reversing diabetes in both rats and clinical trials based on different mechanisms from aerobic glycolysis to Beta cells regeneration. BCG is a multi-face vaccine that has been used extensively in protection from TB and leprosy and has been repositioned for treatment of bladder cancer, diabetes & multiple sclerosis. Recently, the COVID-19 epidemiological study confirmed that universal BCG vaccination reduced morbidity and mortality in certain geographical areas. Countries without universal policies of BCG vaccination (Italy, Nederland, USA) have been more severely affected compared to countries with universal and long-standing BCG policies that have shown low numbers of reported COVID-19 cases. Some countries have started clinical trials that included a single dose BCG vaccine as prophylaxis from COVID-19 or an attempt to minimize its side effects. This proposed research aims to use BCG vaccine as a double-edged weapon countering both COVID-19 & diabetes, not only as protection but also as therapeutic vaccination. The work includes a case study of regenerated pancreatic beta cells based on improved C-peptide & PCPRI laboratory findings after BCG vaccination for a 9 years' patient. The patient was re-vaccinated based on a negative tuberculin test & no scar at the site of injection of the 1st BCG vaccination at birth. Furthermore, the authors in the present article described a prospective BCG multi-dose clinical study in full details that they will apply in case of acceptance of their submitted grant & the ethical committee approval. The aim of the clinical study is to check if double dose BCG (4 weeks apart) will show a significant difference in the protection of health care professionals in Egypt. The authors suggest and invite the scientific community to take into consideration the concept of direct BCG re-vaccination (after 4 weeks) because of the reported gene expressions & exaggerated innate immunity consequently. As the diabetic MODY-5 patient (mutation of *HNF1B*, Val2Leu) was on low dose Riomet® while eliminating insulin gradually, a simple analytical method for metformin assay was recommended to ensure its concentration before use as it is not approved yet by the Egyptian QC labs.

Keywords:
COVID-19; Multi-dose BCG; Beta cells regeneration; Improved C-peptide; Serendipity.

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1. Introduction:

Diabetic patients are more liable to develop serious complications from COVID-19 in a mechanism like its sister virus (MERS-CoV) that binds to the receptor-binding domain of dipeptidyl peptidase IV promoting pulmonary inflammation and macrophage infiltration [1]. Many recent COVID-19 studies reported that diabetes was a major contributing factor either for non-survivals &/or hospitalization, representing 12% of non-survivors [2] and 22% of the hospitalized patients in another study [3]. Generally, people with Diabetes Mellitus are most likely to suffer from different complications when infected with the virus ranging from mild to severe. Diabetic patients with uncontrolled blood glucose are more liable to respiratory tract infection due to their compromised immunity [3-4].

Recent literature has linked and introduced the term “trained immunity” to BCG vaccine. BCG vaccine achieves this effect by inducing pattern recognition receptor NOD2, resulting in metabolic changes with epigenetic rewiring [5]. The reasoning behind the initiating of this process is immunomodulation [6]. This immune-modulatory mechanism of BCG was reported to limit the progression of autoimmune Type 1 Diabetes Mellitus (T1D) and Multiple sclerosis (MS). Tumor necrosis factor (TNF) is a cytokine with pro-inflammatory properties and immune-stimulatory features that is deficient in both TID and MS autoimmune diseases. In T1D, there is an imbalance between overexpression of pathogenic cytotoxic T cells (CD8 CTLs) and a shortage in T-regulatory (CD4 Treg) cells. This pathogenic imbalance could be restored with TNF which leads to proper stimulation of T cell antigen presenting cells and mediates elimination of auto-reactive T cells causing T1D and MS. BCG vaccination is considered a potent TNF inducer that replenishes TNF deficiency [9].

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BCG vaccine possesses a deep-rooted safety profile. Adverse effects are mainly due to inefficient application as deep injection or incorrect dose. Although BCG is known frequently to cause local reactions, anaphylaxis is a rare side effect. It has existed for 80 years now and is one of the most commonly used of all current vaccines with 80% worldwide coverage [10]

We propose using BCG as double-edge weapon for both COVID-19 & diabetes, not only for protection but also as therapeutic vaccination. The work included is a case study of regenerated pancreatic beta cells based on C-peptide & PCPRI laboratory findings after BCG vaccination for a 9 year old patient. Furthermore, the authors in the present study described a prospective BCG multi-dose clinical study in full details that they will apply in case of acceptance of their submitted grant & the ethical committee approval (code CL/2005). The aim of the clinical study is to validate if double dose BCG (4 weeks apart) will provide a significant difference in protection of the Egyptian health care professionals against COVID-19. The authors suggest and invite the scientific community to take into consideration the concept of direct re-vaccination (after 4 weeks) because of the reported changes in genes expressions & exaggerated stimulation of innate immunity.

We assume that BCG had a role in the regeneration of beta cells of pancreas in the underlying case study based on the increase of the postprandial C-peptide value from 1.53 ng/mL (before BCG vaccination) to 4.88 ng/mL after 5 weeks of post BCG vaccination. Recently, the molecular mechanism involving the immune-stimulatory activity of foreign nucleic acids through cytosolic DNA sensing has been progressing. Both cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway are stimulated upon recognition of cytosolic DNA leading to type I interferons stimulation [11]. Type I IFNs is then achieve a reported role in enhancing and promoting pathways of innate immunity, plus its support in developing adaptive immunity *via* increasing pro-inflammatory cytokines like

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IL-12P70 [12]. It was recently reported that up-regulation of some genes contribute in BCG activity as *IL17F* gene with its associated cytokines IL22, IL23A, and *FCGR1B* [13]. Other studies proposed that up-regulation of *ICAMI* gene (Intercellular Adhesion Molecule-1 gene) is responsible of a part of BCG activity. ICAM-1 is a trans-membrane protein important for several basic immunological processes, including antigen presentation to T lymphocytes and leucocytes extravasation. This is especially crucial in case of using BCG as antitumor agent [14].

Finally, as the diabetic case study was on low dose Riomet® while eliminating insulin gradually, a simple analytical method for metformin assay was recommended to ensure its concentration before use as it is not approved yet by the Egyptian QC labs. Some parameters were adjusted according to previous LC work [15] on metformin combinations while the previous LC-MS work on metformin [16] was excluded to avoid its high cost. ICH guidelines [17] were applied for the method validation.

2. Results & discussion

2.1. COVID-19, Diabetes & BCG

Diabetic patients are at higher risk of COVID-19 infection especially poor controlled patients. Diabetes increases mortality in patients infected with H1N1, SARS, and MERS-CoV. Also, diabetes is responsible for 42.3% of 26 COVID-19 fatalities in Wuhan, China [4, 18, and 19]. Recent reports in correspondence to different clinical data shows that fatalities in hypertensive and diabetic patients are higher with COVID-19 infection. COVID-19 virus binds to its target *via* angiotensin-converting enzyme 2 (ACE2) on epithelial cells of the lungs, kidney, blood vessels and intestine. On the other hand, the expression of ACE2 is elevated in type 1 and type 2 diabetic cardiovascular patients who have been long treated with ACE inhibitors and angiotensin II receptor blockers (ARBs) leading to up-regulation of ACE2. Additionally, ACE2 up-regulation may occur *via* using

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thiazolidinediones in type 2 diabetes mellitus. As a result, patients with diabetes and hypertension have higher risk of developing severe presentation and fatal outcome from COVID-19 [20-21]

BCG reported to initiate a memory-like innate immune training rather than its known adaptive immune rule as a vaccine. This recently discovered mechanism is through transcriptional modification on bone marrow hematopoietic stem cells in multipotent progenitors (MPPs). This in turn promotes an expansion and differentiation in hematopoietic stem cells with unintended, yet desirable behavior. Collectively, BCG vaccine would motivate production of bone marrow derived macrophages/monocytes which are minded epigenetically to proceed more protective actions against tuberculosis [22].

In addition to this role in training immunity, BCG is used as a therapy for bladder carcinoma. Clinical studies show its ability to reduce cancer recurrence and further progression, in comparison with other anticancer agents. Being antitumor agent is mainly due to its capacity to promote TNF levels [23]. These studies show the essential need for more understanding of the behavior of those immune trainers chiefly BCG molecularly and clinically which would reveal more data for widening the therapeutic approaches for critical diseases within human defected immunity including possible repurposing as therapeutic vaccination against COVID-19 at least to develop less severe symptoms.

Regarding BCG vaccine safety, there is a small margin of risk, however as BCG vaccine is a live attenuated vaccine, which might lead to infection within 8 to 12 weeks and this must be taken into consideration with immunosuppressed individuals or with infection of HIV. Moreover, BCG over reprograming of the immunity causes more activated CD4 T cells which is convenient target for HIV replication. Less likely BCG contributes to

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dysfunction of cell mediated immunity; phagocytes and IFN-γ mediated pathways. In clinical trials using BCG in treatment of cancer bladder, only 5% of patients were presented with complication as reactive arthritis in large joints due to multiple intravesical administration of the vaccine [24].

However, BCG randomized controlled clinical trials were proved to be the most widely acceptable due to its safety ratios in comparison to other therapeutic agents. This relative supremacy makes it the only infancy vaccine which can be used in clinical trials with long periods of investigation, generally BCG possess a stable abiding positive effect on health [25].

BCG multi-dose clinical trials in Egypt may show different results than those obtained from US volunteers' multi-dose long term study that was targeting diabetes [26] due to ethnic and genetic factors [27-29]. Ethnic difference has been taken in consideration in recent years. Typically, global companies start clinical development in Japan after the United States and Europe, some genetic factors could explain a significant proportion of dose variability of many drugs between different ethnic groups [28-29]. The recognition of racial differences in disease outcome may explain a significant proportion of dose variability. This evidence underpins the prevailing hypothesis that genotype guided therapy should improve dosing accuracy [28-29].

2.2. Case study of beta cells regeneration after BCG by serendipity

Case presentation

A nine years old male patient was admitted to the emergency department with random blood glucose of 580 mg/dL and a history of polyuria, polydipsia without ketoacidosis. Diabetes Mellitus type-1 was suspected and accordingly insulin therapy began. The patient was treated successfully and discharged from the hospital one day later

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on insulin regimen. On discharge, the patient performed the following lab studies; fasting blood glucose and postprandial blood glucose (FBS/PPBS), glycosylated hemoglobin (HbA1c), postprandial C-peptide, complete urine analysis. In addition, Insulin/islet cell/IA2/glutamic acid decarboxylase antibodies, hematological, thyroid, liver, renal functions, electrolytes, CRP, CBC, Anti tissue IgA, IgG, G6PD, TIBC, iron, serum ferritin, vitamin D, food intolerance test, lipid profile and ApoB/ApoA were also recommended due to his parents medical background. At that time, the patient's average blood glucose was 80 to150 mg/dL FBS and 170 to 250 mg/dL PPBS with insulin therapy and multiple correction doses. Ultrasound on the abdomen showed normal size and images for the pancreas, liver, kidneys, spleen, appendix, gall bladder, intestines, urinary bladder & blood vessels of the abdomen. No aneurysm or any other abnormal finding was found in the ultrasound investigation. All laboratory results before BCG intradermal administration are shown in (table 1).

His C-peptide was 1.53 ng/mL, PPBS was 273 mg/dL, HbA1c was 9.6 % and only glutamate decarboxylase antibody was positive (33.5 U/mL). His complete blood picture, thyroid, liver function, kidney function and lipid profile & all the other laboratory investigations were all in the normal range (table 1). Postprandial C-peptide/blood glucose ratio (PCPRI) was calculated and found to be 0.56. Clinical exome sequencing on Illumina Hiseq2500 platform (Illumina Inc., USA) was performed at Generation Lab (Cairo, Egypt). Rare and non-silent variants in 12 maturity-onset diabetes of the young (MODY) genes were investigated for pathogenicity. A rare missense variant (*HNF1B*, Val2Leu) in MODY genes was identified.

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Table 1: Laboratory investigation findings before BCG intradermal administration

Laboratory investigation	Result	Reference range
Glycated Hemoglobin (HbA1c)	9.6 %	4.5 – 5.7, Normal range 5.8 – 6.4, Prediabetic > 6.5, Diabetic 6 – 7, Good control 7 - 8, Fair control >8, Poor control
C-Peptide (postprandial)	1.53 ng/mL	2.7 – 5.6
Plasma glucose 2hrs PP	273 md/dL	70 - 140
Glutamate Decarboxylase Antibodies	33.5 U/mL	Negative if < 10 Positive if > or = 10
IA2 Antibodies	0.16 U/mL	Negative if < 1 U/mL Grey zone if 1 - 2 U/mL Positive if > 2 U/mL
Insulin Antibodies	3.6 %	Negative if < 8.2 Positive if > or = 8.2
Anti-Islet cell Antibodies	Negative	Negative
Anti-tissue transglutaminase IgA	1.1 U/mL	Negative if < 10 Positive if > or = 10
Anti-tissue transglutaminase IgG	0.9 U/mL	Negative if < 10 Positive if > or = 10
C-reactive protein (CRP)	0.1 mg/L	< 5
Homocysteine	14.7 umol/L	3.7 – 13.9
Serum cholesterol	181 mg/dL	Up to 170 Desirable Over 200 High risk
HDL-Cholesterol	74 mg/dL	Up to 40 High risk Over 60 Low risk
LDL	96 mg/dL	Up to 110 Acceptable 110 - 129 Borderline Over 130 High
Non-HDL-Cholesterol	107 mg/dL	Up to 130 Optimal
Serum Triglycerides	57 md/dL	Up to 150 Normal 150 - 199 Borderline 200 - 499 High
Serum VLDL Cholesterol	11 mg/dL	Up to 30
T. cholesterol / HDL Cholesterol	2.45	Less than 4.44
LDL/HDL Cholesterol	1.3	Less than 3.22
Serum Apo A1	190 mg/dL	>120 recommended
Serum Apo B	69 mg/dL	0 – 100 Desirable Over 120 High risk
Serum ApoA1/Apo B	2.75	More than 1
25(OH) Vitamin D	21.28 ng/mL	<20 Deficiency 21-29 Insufficiency 30-100 Sufficiency Over 150 Hypervitaminosis
Serum urea	41 mg/dL	10.8-38.4

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Serum Creatinine	0.74 mg/dL	0.39-0.8
Serum uric acid	2.6 mg/dL	3.5-7.2 mg/dL
ALT (SGPT)	11 U/L	0-50
AST (SGOT)	22 U/L	0-50
Bilirubin (total)	0.34 mg/dL	0.3-1.2
Bilirubin (direct)	0.07 mg/dL	Up to 0.2
A/G ratio	1.47	1-2
Albumin, serum	4.7 g/dL	3.8-5.4
Total protein, serum	7.9 g/dL	5.7-8
TSH	2.893 uIU/mL	0.3-4.5
Free T3	3.61 pg/mL	2.3-5.3
Free T4	1.45 ng/dL	0.77-1.32
PTH	30.4 pg/mL	6-80
Serum calcium (total)	10.2 mg/dL	8.8-10.8
Ionized calcium (Ca^{++})	4.8 mg/dL	4.8-5.5
Serum phosphorus	5 mg/dL	3.2-5.8
Iron, serum	51 ug/dL	50-120
Total iron binding capacity (TIBC)	362 ug/dL	250-400
Serum potassium (K^{+})	4 mmol/L	3.5-5.1
Serum sodium (Na^{+})	139 mmol/L	136-146
Serum magnesium	2.1 mg/dL	1.8-2.6
G6PD Activity (quantitative)	364 U/10^{12} RBCs	221-570
Random albumin-urea	2 mg/dL	Up to 14 mg/dL
N.B. IgE specific for 30 different food allergens did not show any significant reaction (all <0.35 IU/mL). Urine analysis showed no significant findings. CBC showed no pathogenic markers. No ketone bodies, bilirubin, uro-bilirubin &/or albumin were found in urine and its microscopic examination showed normal results. Complete FoodPrint® IgG antibody test for 200 food groups showed positive results (>30 U/mL) for Casein (& all dairy), Gluten (& all wheat) and some nuts.		

BCG Vaccination outcomes

Although BCG vaccination has been compulsory in Egypt since 1974 and the patient was vaccinated directly after birth; the patient has no vaccination scar and tested tuberculin negative [30]. And as the patient has family history of TB, BCG re-vaccination in a Ministry of Health office with the standard dose of 0.05 ml in the lateral upper part of the left shoulder was done [31]. After 2-3 weeks of BCG vaccination, the patient showed many hypoglycemic episodes that was attributed to gradually increase his own insulin secretion. Insulin was tapered and metformin (Riomet®) was introduced. Four weeks after vaccination, metformin was stopped. Five weeks post vaccination, his daily routine

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monitoring of blood glucose for FBS/PPBS was 89 and 153 mg/dl with no insulin therapy and no oral hypoglycemic drugs. Also, the above-mentioned lab studies (before vaccination) were repeated and all results after BCG intradermal administration are shown in (table 2), C-peptide was 4.88 ng/mL and HbA1c was 7%. His postprandial C-peptide/blood glucose ratio (PCPRI) was calculated and found to be 3.2 (100 * 153/4.88)

In 1997 Horikawa et. al. reported the first case of Maturity onset diabetes of the young subtype 5 (MODY5) [32]. We herein report a mutation in the *HNF1B* gene of a 9-years-old Egyptian patient with atypical non autoimmune diabetes phenotype of MODY5 reflecting extensive clinical and genetic heterogeneity of the disease. In patients with mutation of *HNF1B* gene, the primary pathophysiology of diabetes in (Mody5) is characterized by decreased insulin secretion with progressive hyperglycemia due to pancreatic atrophy [33-35]. Accordingly, when our patient was diagnosed with diabetes, insulin treatment was required due to defective insulin secretion associated with pancreatic atrophy [36]. However, upon BCG re-vaccination, after 5 weeks, the patient was near normo-glycemic range with no insulin therapy, so we presumed there was restored insulin secretion due to pancreatic beta cells regeneration.

C-peptide value was increased to 4.88 ng/mL (from 1.53 ng/mL) after vaccination (more than 3 times). Moreover, PCPRIA was increased to 3.2 from 0.56 (more than 5 times). It has been proven that the mechanism of near normal blood sugar restoration following BCG treatment is due to the regeneration of insulin-secreting islets of pancreas [26, 37 and 38]. As previously published, the stimulation in tumor necrosis factor (TNF) after BCG vaccine increases both cytotoxic T cell death and Treg expansion [39-41] . C-peptide is co-secreted with insulin from the pancreas and can be used to selectively detect the secretion of endogenous insulin [42-43]. A comparison between the patient's C- peptide pre-vaccination and post-vaccination of 1.53 ng/ml and 4.88 ng/ml respectively is proof of

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a similar mechanism of pancreatic islet regeneration. Accordingly, we observed the stable lowering of blood sugars after BCG vaccinations; FBS/PPBS is 89 and 153 mg/dl with no insulin therapy compared to FBS/PPBS 80 to150 mg/dl and 170 to 250 mg/dl before BCG vaccination with insulin therapy (0.5 unit/kg/day).

Table 2: Laboratory investigation findings after BCG intradermal administration

Laboratory investigation	**Result**	**Reference range**
Glycated Hemoglobin (HbA1c)	7 %	4.5 – 5.7, Normal range 5.8 – 6.4, Prediabetic > 6.5, Diabetic 6 – 7, Good control 7 - 8, Fair control >8, Poor control
C-Peptide (postprandial)	4.88 ng/mL	2.7 – 5.6
Plasma glucose 2hrs PP	153 md/dL	70 - 140
Fasting plasma glucose (without insulin) (& without oral hypoglycemic drugs)	89 mg/dL	70-100
Blood viscosity	1.8	1.4-1.8
ESR	6 mm	3-12 mm
Serum Amylase	80 U/L	28-100
Lipase level in serum	27 U/L	Up to 60
Serum ferritin	59.9 ng/mL	21.81-274.66
Rheumatoid factor	8.78 IU/mL	Up to 14
C-reactive protein (CRP)	0.3 mg/L	< 5
Homocysteine	10.68 umol/L	3.7 – 13.9
Serum cholesterol	187 mg/dL	Up to 170 Desirable Over 200 High risk
HDL-Cholesterol	72 mg/dL	Up to 40 High risk Over 60 Low risk
LDL	104 mg/dL	Up to 110 Acceptable 110 - 129 Borderline Over 130 High
Non-HDL-Cholesterol	115 mg/dL	Up to 130 Optimal
Serum Triglycerides	56 md/dL	Up to 150 Normal 150 - 199 Borderline 200 - 499 High
Serum VLDL Cholesterol	11 mg/dL	Up to 30
T. cholesterol / HDL Cholesterol	2.6	Less than 4.44
LDL/HDL Cholesterol	1.44	Less than 3.22
Serum Apo A1	192 mg/dL	>120 recommended
Serum Apo B	70 mg/dL	0 – 100 Desirable Over 120 High risk
Serum ApoA1/Apo B	2.74	More than 1
ACTH (am)	28.2 pg/mL	Less than 65

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Serum urea	40 mg/dL	10.8-38.4
Serum Creatinine	0.64 mg/dL	0.39-0.8
Serum uric acid	4.1 mg/dL	3.5-7.2 mg/dL
ALT (SGPT)	16 U/L	0-50
AST (SGOT)	31 U/L	0-50
Bilirubin (total)	0.34 mg/dL	0.3-1.2
Bilirubin (direct)	0.07 mg/dL	Up to 0.2
Alkaline phosphatase	201 U/L	42-362
TSH	3.61 uIU/mL	0.3-4.5
Free T3	3.57 pg/mL	2.3-5.3
Free T4	1.22 ng/dL	0.77-1.32
GH	5.11 ng/mL	Up to 3
Cortisol (9 am)	18.14 µg/dL	4.3-22.4
Serum calcium (total)	10.2 mg/dL	8.8-10.8
Ionized calcium (Ca^{++})	4.9 mg/dL	4.8-5.5
Serum phosphorus	4.7 mg/dL	3.2-5.8
Iron, serum	94 ug/dL	50-120
Serum potassium (K^{+})	3.8 mmol/L	3.5-5.1
Serum sodium (Na^{+})	134 mmol/L	136-146
N.B. Urine analysis showed no significant findings. No ketone bodies, bilirubin, uro-bilirubin &/or albumin were found in urine and its microscopic examination showed normal results.		

Therefore, we concluded that the BCG re-vaccinations did induce a clinically meaningful return of C-peptide levels in the pancreas by regeneration, as reflected on the patient's blood glucose level & postprandial C-peptide levels.

It is worthy to mention that some other lab investigations were requested by the parents to exclude secondary diabetes &/or pancreatitis and to check the overall health of the patient and all of them showed good results in the normal range including ESR, blood viscosity, ACTH, Cortisol, GH, Rheumatoid factor, Amylase and Lipase. All the results for the previously mentioned laboratory investigations are shown in (table 2).

BCG re-vaccination after four weeks was used by kuhtreiber *et al.* in his work published at NPJ vaccines [26] with one of the most interesting outcomes of multi-dose BCG human clinical trials suggesting aerobic glycolysis as the mechanism of action for BCG anti-hyperglycemic effect. BCG re-vaccination (after 4 weeks) resulted in

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demethylation of regulatory T cell signature genes *in vivo* with enhanced mRNA expression [26]. BCG effects on the gene level are interesting especially regarding the part related to "innate or trained" immunity. The authors not only recommend multi-dose BCG clinical trials for COVID-19 in Egypt, but also they invite the scientific community and the already ongoing studies with single dose BCG to go through another dose after 4 weeks form the start of the study. So, the frequency of BCG vaccination is critical.

Double dose BCG did not show complications and it is well tolerated with high safety profile & it had been proved to initiate a cycle of gene modifications that may be the key against the current fight with COVID-19 outbreak. Single dose BCG may not be enough for the fight against the aggressive COVID-19 and many doses (more than 2) may result in complications as the case with multiple dose intravesical BCG for bladder cancer (although different route of administration). So the authors suggest at least 2 doses (4 weeks apart) as that showed already high safety profile in previous human study targeting diabetes and to ensure BCG ability to re-modulate immunity towards our desired protective &/or treatment activity in case of cytokine storm syndrome of the most recent COVID-19. Furthermore, the complications resulted from frequent multi-dosing of BCG as antitumor agent mainly in bladder carcinoma are different as direct agent responsible of tumor cells apoptosis in that protocol. Finally, many countries like Brazil for example are still recommend BCG re-vaccination to protect people from TB. So, what if re-vaccination also reverse diabetes & protect from COIVD-19 as double-edge weapon especially for the first line health care workers.

Assay of metformin in Riomet® oral solution

As the diabetic case study was on low dose Riomet® while eliminating insulin gradually, a simple analytical method for metformin assay was recommended to ensure its

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concentration before use as it is not approved yet by the Egyptian QC labs. Thermo Fisher UPLC (Ultimate 3000, USA), Symmetry® C_{18} column (100 mm × 2.1 mm, 2.2 µm) & Diode Array detector (3000RS, USA) were used with mobile phase (methanol: water, 50:50, *v/v*) in the isocratic mode at 237 nm. The flow rate was 0.1 mL min^{-1} and the injection volume were 10 µL. Calibration curve was obtained by plotting Area under the peak against the corresponding concentrations (2 - 50 µg/mL). Good UPLC peak was obtained (figure 1). The regression equation was found to be (AUP = 1.6664 Conc. + 1.5789, r^2 = 0.9999) where AUP is the area under the peak and r^2 is the regression coefficient. LOD (limit of detection) and (limit of quantification) were found to be (0.63 µg/mL) and (1.91 µg/mL), respectively. STEYX (residual standard deviation of the regression line) was found to be 0.319. S_b (standard deviation of the slope) & S_a (standard deviation of the intercept) were found to be 0.00785 & 0.29104, respectively. Confidence intervals for slope & intercept were found to be (1.6664 ± 0.0131) & (1.5789 ± 0.459), respectively.

The developed UPLC-UV for metformin assay was validated successfully according to ICH guidelines [17]. Metformin concentrations (5, 25, and 45 µg/mL) were assayed three times within the same day to assess the intra-day precision while inter-day precision was assessed on three successive days. Metformin concentrations were calculated using the corresponding regression equation to check the accuracy of the method with n = 3 using recovery percent (R %) and it was found to be 99.56 ± 1.89 (mean ± standard deviation). To check the precision, both intra-day and inter-day percent relative standard deviation (% RSD) were calculated and showed values below 2%

Regarding Riomet® application, the resultant mean of recovery percent ± standard deviation of three determinations was equal to 96.24 ± 1.33. Riomet® inactive ingredients did not show interference confirming the specificity of the method including hydrochloric acid, propylene glycol, glycerin, potassium bicarbonate, sucralose, xylitol. Regarding the

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system suitability tests, the number of theoretical plates was found to be 1406 while the tailing factor of the peak was found to be 1.01. In conclusion, the simple proposed UPLC method was proved to be suitable for determination of metformin in bulk and in Riomet® in a reasonable run time and the obtained Riomet® recovery was optimum and suitable for use by the patient in the case study.

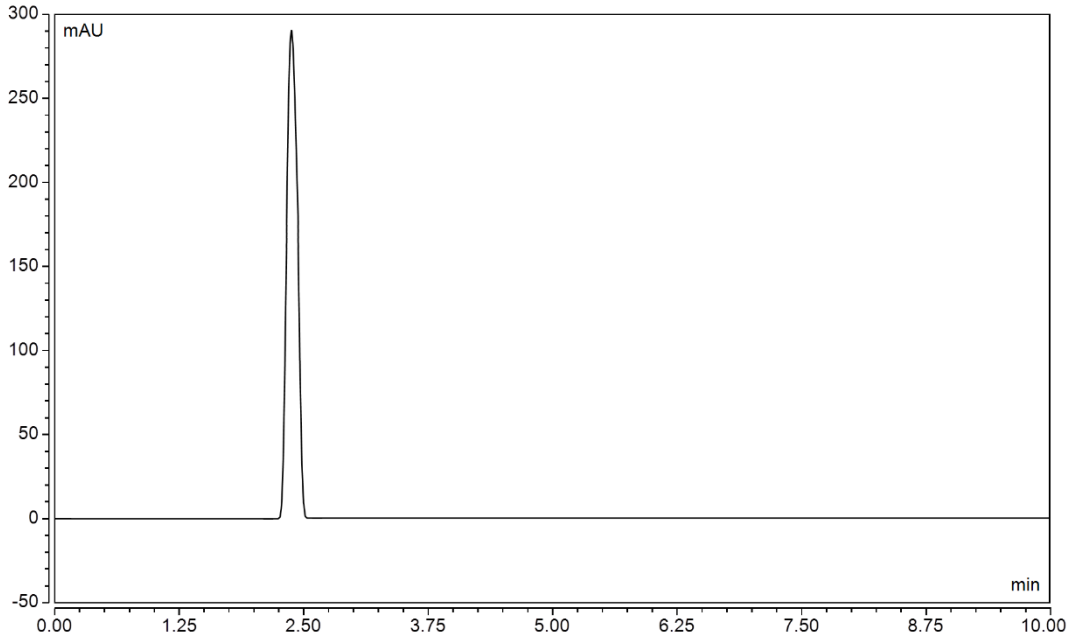

Figure (1): UPLC-UV Chromatogram of metformin hydrochloride (30 µg/mL)

2.3. Prospective clinical trial (multi-dose BCG for COVID-19)

Subjects and study design

A randomized clinical trial will be conducted to test the use of multi-dose BCG vaccine (intradermal, 4 weeks apart) as a prophylaxis from Covid-19 for 150 Egyptian healthcare professionals (75 as control). No placebo will be used, since it is unlikely to mimic the pustule developed following a BCG vaccination, hence the health care volunteers were not be blinded to the intervention. However, test and control volunteers examined by a plaster and blood samples were known by anonymized study id numbers.

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Criteria of Selection

Regarding the inclusion criteria, healthy volunteers from the health care professionals will be included (between 20 & 50 years old). Female participants should not be pregnant and should avoid pregnancy throughout the 3-month study. All participants will sign an informed consent. All health care volunteers will be presented with the material related to this study and a copy of consent for consideration as well as contact information of research staff available to answer any questions or concerns.

Regarding the exclusion criteria, participants are not eligible for the study if they have an acute illness or body temperature ≥37.5 degree Celsius. Participants who have a history or evidence of any autoimmune disease, active tuberculosis, allergic disease, immunosuppressive medication, immunoglobulin, blood products, or any chronic illness will be excluded. Pregnant females will be excluded from the study. Participants that test COVID-19 positive before the first BCG vaccination will be excluded from the study.

Study Groups

75 subjects will receive intradermal BCG vaccine (2 doses, 4 weeks apart) as 0.05 ml (20×10^6 colony forming units/ml) from a single dose plastic syringe through a short beveled 10 mm 25-gauge intradermal needle, into the lower deltoid region of the right arm at the insertion point of the muscle (as group A) while the other 75 subjects will be considered as control (as group B). Protocol was submitted for approval by the Ethical Committee Board at The British University in Egypt (code CL/2005). The studies will be conducted in accordance with the Declaration of Helsinki and applicable local regulations for conducting clinical trials on medicinal products in humans.

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Outcomes Measures

Primary Endpoint:

BCG will be assessed for its ability to mitigate the prevalence and severity of Covid-19 symptoms. Blood samples, nasopharyngeal and oropharyngeal swab and sputum samples will be collected from all participants (both test & control) before the first BCG vaccination and at 1, 2 and 3 months after vaccination. At 1 month, all the test group will be re-vaccinated by BCG. About 10 mL of whole blood will be drawn from each participant and kept in a heparin treated tube to prevent coagulation, and transported to Ain shams Specialized Hospitals and El Demerdash Hospital Central Labs for testing SARS-CoV-2 by real-time reverse transcriptase (RT)-PCR, IgG antibodies, IgM antibodies, highly sensitive CRP, Liver enzymes (SGPT, SGOT), D-Dimer, Serum ferritin, ESR, CBC differentiated that should include the following, Leukocyte count, Lymphocyte count, Neutrophil-lymphocyte-ratio (NLR) and lymphocyte subsets (Helper T cells CD3+CD4+ & Suppressor T cells CD3+CD8+) in addition to CT scan. Participants that test COVID-19 positive before the first BCG vaccination will be excluded from the study.

Both Group A (test) & Group B (control) will be monitored for degree of clinical manifestations in correlation to lab findings. RNA will be extracted from clinical samples with a QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany). All specimens will be handled under a biosafety cabinet according to laboratory biosafety guidelines of Ain shams University Central Labs for Control and Prevention for COVID-19. According to the protocol issued by the National Institute for Viral Disease Control and Prevention in China [44], two sets of primers will be used for two target genes open reading frame 1ab [ORF1ab] and nucleocapsid protein [N]. RT-PCR assay will be performed using a 2019-nCoV nucleic acid detection kit. A cycle threshold

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value (Ct-value) less than 37 was defined as a positive test result, and a Ct-value of 40 or more was defined as a negative test. A medium load, defined as a Ct-value of 37 to less than 40, required confirmation by retesting [45].

Secondary Endpoint:

The secondary outcomes are the adverse events, including local and systemic ones related to vaccination, admissions to hospital and mortality. Safety of the BCG vaccine treatment regimens were based on the induction of adverse events that was graded according to FDA toxicity tables for Healthy Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials [45]. We will record adverse events during the first 30 days after each vaccination i.e. study days 0–30, 31–60, and during the 3-month study period (61-90).

Novelty of the suggested multi-dose BCG clinical trial for COVID-19

As reported by the first author in a preprint submitted as a letter to the editor, BCG Vaccine repositioning to COVID-19 is a cost-effective alternative to the traditional vaccine approach as BCG induces non-specific protection as innate immune cells, including monocytes and natural killer cells. It protects from TB and Leprosy while it is suggested to treat bladder cancer and diabetes by turning on immunity and it resets the epigenetic programming of some genes and immunity related markers [46]. The author recommends that COVID-19 vaccination clinical trials should consider multiple doses of BCG as some preliminary studies suggested BCG to fight COVID-19 but they did not consider the use of multiple intradermal BCG vaccination (at least 2 doses, 4 weeks apart) for the prophylaxis (&/or treatment) of COVID-19 [46]. Recently, COVID-19 epidemiological study confirmed that universal BCG vaccination reduced morbidity and mortality in certain geographical areas [47]. Countries without universal policies of BCG vaccination (Italy, Nederland, USA) have been more severely affected

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compared to countries with universal and long-standing BCG policies that showed reduced number of reported COVID-19 cases [47]. The combination of reduced morbidity and mortality makes BCG vaccination a potential new tool in the fight against COVID-19. Some countries started a clinical trial that included single dose BCG vaccine as a prophylaxis from COVID-19 or at least they predict the vaccinated health care professional may develop less severe symptoms if got infected [46].

3. Authors' contribution

Bassam Ayoub suggested the work as the Principal Investigator (PI) for the present work & the prospective research grant dealing with repurposing of multi-dose BCG targeting both COVID-19 & Diabetes (type-1, type-2, MODY). Bassam Ayoub suggested the BCG multi-dose for COVID-19 either as prophylaxis or as a treatment after acceptance of his letter to the editor discussing multi-dose BCG. Eman Ramadan as the Co-PI participated in the work frame & main ideas. All the authors including the PI & Co-PI participated in the literature review, commented on the case study findings, worked on the clinical trial suggestion with all details, participated in writing the manuscript & revised the whole study findings. Bassam Ayoub & Mariam Tadros conducted the analysis part preliminary investigations, LC method development and its validation, collected, analyzed the data with equal contribution.

4. Competing financial interests

Authors declare NO financial competing interests

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