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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

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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 HbA_{1c} and/or irregular blood glucose levels. Diabetic patients' mortality rates with COVID-19 are higher than those of cardiovascular or cancer patients. Recently, Bacillus Calmette-Guérin (BCG) vaccine 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 tuberculosis (TB) and leprosy and has been repositioned for treatment of bladder cancer, diabetes and multiple sclerosis. Recently, COVID-19 epidemiological studies 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 and 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 and PCPRI laboratory findings after BCG vaccination for a 9 year old patient. The patient was re-vaccinated based on a negative tuberculin test and no scar at the site of injection of the 1st BCG vaccination at birth. 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 and 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.

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 (Kulcsar et al. 2019). Many recent COVID-19 studies reported that diabetes is a major contributing factor either for non-survivals and/or hospitalization, representing 12% of non-survivors (Zhang et al. 2020) and 22% of the hospitalized patients in another study (Yang et al. 2020). 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 (Gupta et al. 2020; Mourits et al. 2018).

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 (Dow 2020). The reasoning behind the initiating of this process is immunomodulation (Fu et al. 1998). 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 (Hayashi and Faustman 1999). 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 (Ristori et al. 2018).

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 to frequently cause local reactions, anaphylaxis is a rare side effect. It has existed for 80 years and is one of the most used of all current vaccines with 80% worldwide coverage (Kowalewicz-Kulbat and Locht, 2017).

We propose using BCG as a double-edge weapon for both COVID-19 and 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 and PCPRI laboratory findings after BCG vaccination for a 9-year-old patient. 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 and 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 5 weeks after 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' (INF) stimulation (Sun et al. 2013). Type I IFNs 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 IL-12P70 (Thada et al. 2020). It was recently reported that upregulation of some genes contribute in BCG activity as *IL17F* gene with its associated cytokines IL22, IL23A, and *FCGR1B* (Matsumiya et al. 2015). Other studies proposed that upregulation of *ICAMI* gene (Intercellular Adhesion Molecule-1 gene) is responsible for 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 the case of using BCG as antitumor agent (Rook et al. 2005).

Finally, as the diabetic case study patient 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 (Ayoub 2015) on metformin combinations while the previous LC-MS work on metformin (Ayoub and Mowaka 2017) was excluded to avoid its high cost. ICH guidelines (*Validation of Analytical Procedures, International Conference on Harmonization (ICH), 2005*, n.d.) were applied for the method validation.

2. Investigations, results and discussion

2.1. COVID-19, diabetes and BCG

Diabetic, especially poorly controlled patients are at higher risk of COVID-19 infection. Diabetes increases mortality in patients infected with H1N1, SARS, and MERS-CoV. Also, diabetes was responsible for 42.3% of 26 COVID-19 fatalities in Wuhan, China (Deng and Peng 2020; Gupta et al. 2020; Song et al. 2019). Recent reports in correspondence to different clinical data shows that fatality rates 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 upregulation of ACE2. Additionally, ACE2 upregulation may occur *via* using thiazolidinediones in type 2 diabetes mellitus. As a result, patients with diabetes and hypertension have a higher risk of developing severe presentation and fatal outcome from COVID-19 (Fang et al. 2020; Wan et al. 2020).

BCG is 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 that are minded epigenetically to proceed more protective actions against tuberculosis (Kaufmann et al. 2018).

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 an antitumor agent is mainly due to its capacity to promote TNF levels (Chou et al. 2017). These studies show the essential need for a better 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, BCG vaccine is a live attenuated vaccine, which might lead to infection within 8 to 12 weeks. 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 a convenient target for HIV replication. Less likely, BCG contributes to dysfunction of cell mediated immunity; phagocytes and IFN-γ mediated pathways. In clinical trials using BCG the in treatment of bladder cancer, only 5% of patients were presented with complications as reactive arthritis in large joints due to multiple intravesical administration of the vaccine (Yamazaki-Nakashimada et al. 2020).

However, BCG randomized controlled clinical trials were proved to be most widely acceptable due to their safety ratios in comparison to other therapeutic agents. This relative supremacy makes it the only infancy vaccine that can be used in clinical trials with long periods of investigation, and in general, BCG possesses a stable abiding positive effect on health (Kristensen et al. 2000).

BCG multi-dose clinical trials in Egypt may show different results than those obtained from a US volunteers' multi-dose long term study that was targeting diabetes (Kühtreiber et al. 2018) due to ethnic and genetic factors. Ethnic differences have 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. 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 (Ayoub et al. 2017; Fukunaga et al. 2011; Limdi et al. 2015).

2.2. Case study of beta cells regeneration after BCG

2.2.1. Case presentation

A nine year 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 on insulin regimen. On discharge, the following lab studies were performed; fasting blood glucose and postprandial blood glucose (FBS/PPBS), glycosylated hemoglobin (HbA_{1c}), 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 and 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.

Table 1: Laboratory investigation findings before BCG intradermal administration

Laboratory investigation	Result	Reference range
Glycated hemoglobin (HbA_{1c})	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-cholesterol	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
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 and/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 (and all dairy), gluten (and all wheat) and some nuts.

His C-peptide was 1.53 ng/mL, PPBS was 273 mg/dL, HbA_{1c} 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 and 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.

2.2.2. BCG Vaccination outcomes

Although BCG vaccination has been compulsory in Egypt since 1974 and the patient had been vaccinated directly after birth, the patient had no vaccination scar and was tested tuberculin negative (Madkour and Khalifa 1977). And as the patient has family history of TB, BCG re-vaccination (Danish 1331 strain) 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 (Davids et al. 2006). BCG vaccination, the patient showed many hypoglycemic episodes that were attributed to gradually increased own insulin secretion. Insulin was tapered and metformin (Riomet®) was introduced. Four weeks after vaccination, metformin was stopped. Five weeks after vaccination, his daily routine 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, All results are shown in Table 2. C-peptide was 4.88 ng/mL and HbA_{1c} was 7%. His PCPRI was found to be 3.2 (4.88/153 * 100).

In 1997 Horikawa et al. reported the first case of maturity onset diabetes of the young subtype 5 (MODY5). 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 the *HNF1B* gene, the primary pathophysiology of diabetes in MODY5 is characterized by decreased insulin secretion with progressive hyperglycemia due to pancreatic atrophy (De Vas et al. 2015; El-Khairi and Vallier 2016; Fajans and Bell 2011). Accordingly, when our patient was diagnosed with diabetes, insulin treatment was required due to defective insulin secretion associated with pancreatic atrophy (Kato et al. 2018). However, upon BCG re-vaccination, after 5 weeks, the patient was near normo-glycemic with no need for insulin therapy, so we presume that insulin secretion was restored due to pancreatic beta cells regeneration.

C-peptide value increased to 4.88 ng/mL (from 1.53 ng/mL) after vaccination (more than 3 times). Moreover, PCPRIA 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 the pancreas (Kodama et al. 2003; Kühtreiber et al. 2018; Ryu et al. 2001). As previously published, the stimulation of TNF after BCG vaccine increases both cytotoxic T cell death and Treg expansion (Ban et al. 2008; Faustman et al. 2012; Okubo et al. 2013). C-peptide is co-secreted with insulin from the pancreas and can be used to selectively detect the secretion of endogenous insulin (Leighton et al. 2017; Wang et al. 2012). 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 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 to 150 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 (HbA_{1c})	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) (and 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-cholesterol	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
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 and/or albumin were found in urine and its microscopic examination showed normal results.

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

It is worth mentioning that some other lab investigations were requested by the parents to exclude secondary diabetes and/or pancreatitis and to check the overall health of the patient and all tests showed good results in the normal range including ESR, blood viscosity, ACTH, cortisol, GH, rheumatoid factor, amylase and lipase.

BCG re-vaccination after 4 weeks was used by Kühtreiber et al. in their work 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 demethylation of regulatory T cell signature genes *in vivo* with enhanced mRNA expression (Kühtreiber et al. 2018). 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 invite the scientific community and the already ongoing studies with single dose BCG to go through another dose after 4 weeks from 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. Also 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 two) may result in complications as is the case with multiple dose intravesical BCG for bladder cancer (although different route of administration). So the authors suggest at least two 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 and/or treatment activity in the case of cytokine storm syndrome of the most recent COVID-19. Furthermore, the complications resulting 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 recommending BCG re-vaccination to protect people from TB. So, what if re-vaccination also reverses diabetes and protects from COIVD-19 as a double-edge weapon especially for the first line health care workers?

2.3. Assay of metformin in Riomet® oral solution

As the diabetic case study patient 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. Thermo Fisher UPLC (Ultimate 3000, USA), Symmetry® C_{18} column (100 mm × 2.1 mm, 2.2 µm) and 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 was 10 µL. Calibration curve was obtained by plotting the area under the peak against the corresponding concentrations (2 - 50 µg/mL). A good UPLC peak was obtained (Fig.). The regression equation was:

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 LOQ (limit of quantification) were 0.63 µg/mL and 1.91 µg/mL, respectively. STEYX (residual standard deviation of the regression line) was 0.319. S_b (standard deviation of the slope) and S_a (standard deviation of the intercept) were 0.00785 and 0.29104, respectively. Confidence intervals for slope and intercept were 1.6664 ± 0.0131 and 1.5789 ± 0.459, respectively.

The developed UPLC-UV method for metformin assay was validated successfully according to ICH guidelines (*Validation of Analytical Procedures, International Conference on Harmonization (ICH), 2005*, n.d.). Metformin concentrations (5, 25, and 45 µg/mL) were assayed 3 times within the same day to assess the intra-day precision and inter-day precision was assessed on 3 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 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 3 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, and xylitol. Regarding the system suitability tests, the number of theoretical plates was 1406 and the tailing factor of the peak was 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.

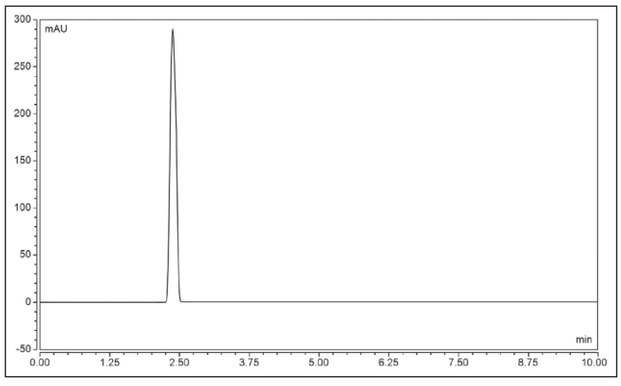

Fig.: UPLC-UV Chromatogram of metformin hydrochloride (30 µg/mL)

2.4. Conclusion: Novelty of the suggested multi-dose BCG for COVID-19

As reported by the first author in a a letter to the editor (Ayoub 2020), BCG vaccine repositioning to COVID-19 is a cost-effective alternative to the traditional vaccine approach because BCG induces non-specific protection as innate immune cells, including monocytes and natural killer cells. It protects from TB and leprosy and 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. The author recommends that COVID-19 vaccination clinical trials should consider multiple doses of BCG because 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 (and/or treatment) of COVID-19. Recently, 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 showed reduced number of reported COVID-19 cases (Miller et al. 2020). The combination of reduced morbidity and mortality makes BCG vaccination a potential new tool in the fight against COVID-19. Some countries started clinical trials that include 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 they become infected (Ayoub 2020).

Authors' contributions: Bassam Ayoub suggested the work as the Principal Investigator (PI) for the present work and the prospective research grant dealing with repurposing of multi-dose BCG targeting both COVID-19 and 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 and main ideas. All the authors including the PI and Co-PI participated in the literature review, commented on the case study findings, participated in writing the manuscript and revised the whole study findings. Bassam Ayoub and Mariam Tadros conducted the analysis part preliminary investigations, LC method development and its validation, collected, analyzed the data with equal contribution.

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