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# Assessment the Possible Effects of Four Lyoprotectants on Cytotoxic Activity of Freezed-dried Intravesical Immune BCG Against EJ138 and 5637 Cell lines

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## Abstract

Immunotherapy of superficial bladder cancer by Mycobacterium bovis Calmette-Guerin (BCG) bladder instillation is used worldwide. Impaction of four lyoprotectant including trehalose, lactose, dextran and monosodium glutamate on changing in the effectiveness of BCG has been studied by measuring its cytotoxicity by the use of amido black staining. The results showed that the cytotoxicity of BCG bacilli at different concentrations against EJ138 and 5637 cell lines were independent of these lyoprotectants.

**Keywords:** Interavesical, BCG, Lyoprotectants, Cytotoxicity.

## Introduction

*Summary*

Approximately 70% of all bladder cancer cases are in the form of early phase or superficial bladder cancer (SBC). While efficacy of chemotherapy is estimated to be 15%; immunotherapy in the form of instillation of Bacillus Calmette-Guerin (BCG) generally has proven more efficacious than chemotherapy (P.-U, 2003)

The antitumor effects of BCG vaccine in treatment of SBC were described as a non-specific biological immune response modifier (A. Bissoyi et al., 2016; F. Mavandadnejad et al., 2013; E. Orihuela et bal., 1987).

Despite the stages of the bladder tumor cells and its impressibility; kinds of BCG strain, its dose; installation protocol, close contacting with the tumor cells, excipients and other additives might be of BCG impresser and its tumor cell toxicity effects apparently. Between the repugnant reports for the best effective BCG strain, some of them showed the strains of 1173P2 and 1331 danish are more invasive than the other ones (Sefidi et al., 2017), while on the contrary, (Gsponer et al., 2012) showed significantly better survival of patients treated with BCG Connaught as compared to BCG Tice and some reports revealed no significant difference in their antitumor activity (P.-U, 2003). The effective dose and installation protocol is still being investigated although the first is proven to be ranged between $10^7$ to $10^{10}$ colony-forming units (CFU)( Yalcinkaya et al., 1998; D.-S. Yu et al., 2015). Definitely close contact between tumour cells and BCG lead to a better tumour suppression activity (Moore et al., 1973).

Excipients as a group of heterogeneous materials are essential components of most of drug products. Safety data of the proposed exposure to the excipients may be needed before marketing. Food and Drug Administration (FDA) has adopted guidance for industry, "Nonclinical Studies for Development of Pharmaceutical Excipients," which focuses on issues associated with development of safety databases that will support clinical use of excipients in drug products (Nafchi et al., 2018; Sefidi et al., 2017). Between protective excipients; trehalose, lactose, dextran and monosodium glutamate (MSG) are being used widely as the stabilizer in some kinds of lyophilized vaccines (Lee et al., 2013; Sefidi et al., 2017).

The objective of the present work was study of possible impact of these four kinds of stabilizer on cytotoxic activity of lyophilized intravesical BCG sub-strain Pasteur 1173P2 formulated with them.

Efficacy of interavesical BCG could be assessed by whether cytopathology study, cytotoxicity assay, detecting cytokines such

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as IFN-γ, TNF-α, Il-1, IL-6 and so on (Elsässer-beileet al., 2005; Taniguchi et al., 1999).

## Material and Methods

*Mycobacterium bovis BCG* of sub-strain Pasteur 1173-P2 was provided by Pasteur Institute of Paris. Trehalose dehydrate (99.5%), sucrose (99.5%), lactose monohydrate (98%), glucose (99.5%), l-glutamic acid monosodium (98%), glycerin (85%), dextran-40 and other chemical substances were all purchased from Sigma Aldrich Co.

Bladder human cell lines (EJ138 and 5637), Culture media and solutions brought to us by Pasteur Institute of Iran.

*Preparation of lyophilized BCG samples*

Lyophilized immune BCG (120mg/vial) containing 0%, 2.5%, 5% and 10% of lactose, trehalose, dextran-40 and monosodium L-glutamate (MSG) were prepared as explained by Sefidi, Kaghazian (Sefidi et al., 2017). A vial of freeze-dried working seed lot of BCG vaccine was reconstituted and inoculated on the surface of Sauton medium following incubation at 37ºC for 20 days. The proliferated bacilli in form of pellicle were passaged 2 times more on the surface of Sauton media. Bacterial pellicle was collected from the Sauton media and was passed through the filtration system to obtain a semi dry biomass. Biomass was weighted and suspended by adding 1.5% L-glutamate solution to obtain the final bulk of vaccine (40 mg/ml). Lyoprotectants were added to the bulk prior to lyophylization.

*Potency test*

Viability was measured by counting the Colony forming unit (CFU) of the samples by spreading their dilution onto the Lowein Stein Junsom culture media following incubation at 37˚C for 28 days (Sefidi et al., 2017).

*Skin sensitivity reaction test*

According to WHO TRS No.771- annex 12, six healthy guinea pigs (250-400 g) with no history of any antibiotic intake were used. Randomly, 1mL of each samples of reconstituted lyophilized BCG was injected intradermal. The size of each lesion was measured after 4 weeks (WHO Expert, 1990).

*Cytotoxicity assay*

EJ138 and 5637 cell lines were propagated in T-flask (25cm$^2$) with RPMI 1640 contained 10% BSA and 400 µg/ml geneticin (G418, Sigma) at 37˚C, 5% CO2. Fresh confluent cells were released by trypsinization and harvested by centrifugation (Schulz ET AL., 1994). The cells were resuspended in fresh culture media and 100 µl of each suspension were dispensed into the wells (30,000 cells /well) in flat-bottom 96-well microplates (Falcon, Germany). Microplates were incubated at 37˚C, 5% $CO_2$ for 24 hours when the cells spread confluent. Spent culture media was refreshed by 100µL of similar one contained 1 to 2mg/mL (approximately 1.5 to 3×10$^7$cfu/mL) of reconstituted of dispersed BCG bacilli and 0.15, 0.3 and 0.6% of the lyoprotectant substances. Blank wells were contained only the cells with and without the lyoprotectants. After 48 hours exposure; cellular sensitivity was assayed using the protein dye amido black 10B (Sigma-Aldrich) to determine viable cell number in vitro (Schulz ET AL., 1994). In this step and prior to staining, treated adherent cells were fixed for 15 minutes by adding 100 µl/well of 10% formaldehyde in 0.1 M sodium acetate/1.5 M acetic acid pH 3.5 then the supernatant was attentively aspirated. For staining of the cells, 100µl of 0.1% amido black staining solution (100 mg amido black dissolved in 100 ml of sodium acetate buffer and filtered) were added to each well for 30 min and were subsequently aspirated. Destaining and removing unbounded dye were carried out by washing the wells once with 150 µl of acidic water (acidified with HC1 to pH 3.5-5). The protein-bound dye was eluted by adding 150 µl of 50 mM NaOH per well and homogeneously dissolved by shaking the plate. Absorbance measurement of the alkaline amido black solution of the wells was performed at the optical density (OD) of 620nm. The mean values of the appropriate replicates were calculated.

*Morphology study*

Microphotographs of amido black-stained cells were taken, using the inverse microscope (Nikon, USA) with a camera system.

## Results

A density-response curve has been observed for different cell density of the EJ137 and 5637 cell lines after staining with amido blak. The OD of eluted amido black corresponded well to the cell numbers so that linearity between OD and cell number were exactly held up to 30,000 of 1534 and EJ138 cells per well.

Table 1. Effect of 4 kinds of lyoprotectants on cytotoxicity of BCG against EJ138 and 5637 cell lines

| sample | Lyoprotectant (%) | BCG (1mg/ml) | (cells×1000) | | cells viability% | |
|---|---|---|---|---|---|---|
| | | | EJ138 | 5637 | EJ138 | 5637 |
| Blank 1 | 0 | 0 | 30.0±1.5 | 30.0±1.3 | 100 | 100 |
| Blank 2 | 0 | 1 | 22.0±1.5 | 20.6±2.1 | 73 | 69 |
| Blank3 | 0 | 2 | 10.1±0.5 | 8.4±0.4 | 34 | 28 |
| Blank4 (MSG) | 2.5 | 0 | 29.8±1.6 | 29.8±1.9 | 99 | 99 |
| Blank5 (MSG) | 5 | 0 | 29.5±1.2 | 29.3±1.5 | 98 | 98 |
| Blank6 (MSG) | 10 | 0 | 29.3±1.9 | 29.0±1.5 | 98 | 97 |
| Lactose | 2.5 | 1 | 21.1±1.0 | 20.3±1.3 | 70 | 68 |
| | | 2 | 8.8±0.4 | 7.8±0.4 | 29 | 26 |
| | 5 | 1 | 21.0±1.3 | 20.1±1.5 | 70 | 67 |
| | | 2 | 8.7±0.3 | 7.8±0.4 | 29 | 26 |
| | 10 | 1 | 20.8±1.1 | 19.7±1.4 | 69 | 66 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | 2 | 8.1±0.3 | 7.1±0.1 | 27 | 24 |
| Trehalose | 2.5 | 1 | 21.5±1.7 | 21.0±1.4 | 72 | 70 |
| | | 2 | 9.1±0.7 | 8.7±0.6 | 30 | 29 |
| | 5 | 1 | 21.2±1.5 | 20.8±0.1.2 | 71 | 69 |
| | | 2 | 9.1±0.09 | 8.9±0.4 | 30 | 30 |
| | 10 | 1 | 21.2±1.0 | 20.7±2.4 | 71 | 69 |
| | | 2 | 8.7±0.6 | 8.2±0.09 | 29 | 27 |
| MSG | 2.5 | 1 | 21.8±1.1 | 21.6±1.9 | 73 | 72 |
| | | 2 | 9.4±0.3 | 9.4±0.8 | 31 | 31 |
| | 5 | 1 | 21.3±1.6 | 21.4±1.6 | 71 | 71 |
| | | 2 | 9.5±0.5 | 9.4±0.3 | 32 | 31 |
| | 10 | 1 | 21.1±1.9 | 21.1±1.7 | 70 | 70 |
| | | 2 | 8.8±0.1 | 8.7±0.2 | 29 | 29 |
| Dextran | 2.5 | 1 | 22.1±2.1 | 22.0±1.5 | 74 | 73 |
| | | 2 | 10.4±0.7 | 9.9±0.6 | 35 | 33 |
| | 5 | 1 | 22.2±0.9 | 21.9±1.1 | 74 | 73 |
| | | 2 | 10.1±0.6 | 9.7±0.5 | 34 | 32 |
| | 10 | 1 | 21.9±1.9 | 21.1±1.4 | 73 | 70 |
| | | 2 | 9.8±0.2 | 9.2±0.3 | 33 | 31 |

Standard deviation was calculated for 3 repeats of each sample

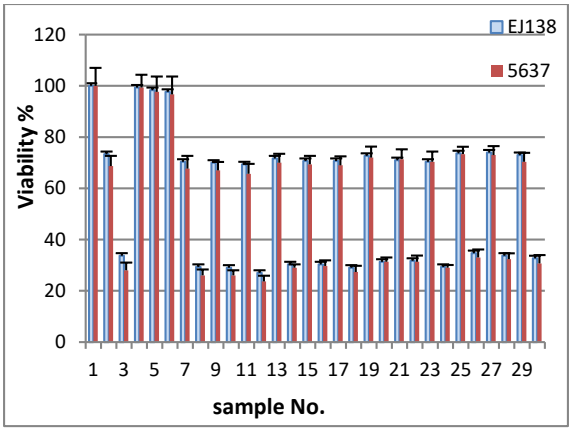


**Figure 1.** Comparison of EJ138 and 5637 in terms of sensitivity to various formulation of BCG

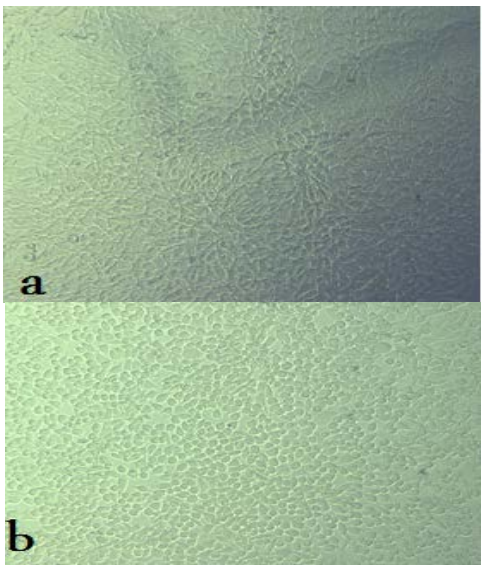


**Figure 2.** the appearance of WJ137 cells (a) before treatment and (b) when treated by 2mg/ml of Bacillus Calmett Guerine (BCG) without amido black staining.

As shown in Table 1 the significant ($P<0.05$) losing of cell viability would be observed in the samples contained BCG in compare to the blank sample (B1) (without BCG) or the samples only contained the lyoprotectants (B4-6). In Fig. 1, the cytotoxicity of BCG against EJ137 was obviously demonstrated. Cytotoxicity effects of BCG against both of the cell lines are very close together significantly ($p<0.01$). Obviously, there is a positive correlation ($r=0.99$) between the cfu of BCG and its cytotoxicity effect on both of the cell lines as achieved by the B2 and B3 samples. There were not noteworthy signs of higher sensitivity of 5637 to BCG than for EJ137 although it is rarely sensed. On the other hand viability of the cells was not changed significantly ($p<0.05$) between the lowest and the highest levels of MSG in B4, B5 and B6 that revealed no cytotoxicity action of MSG represented in the intended concentrations. Although the results were very close together between the substances in terms of cytotoxicity, but as shown in table1 and Fig. 1, the cytotoxicity effect of them can be assumed in the order of dextran< MSG< trehalose< lactose. Results also shown that all of the substances in minimum and maximum concentrations did not lead to a significant alteration in effectivity of BCG against the cell lines in compare to the B2 and B3 samples that revealed the cytotoxicity ability of the mycobacterium BCG has not been affected by the present of the substances at different concentration that means viability of BCG bacteria and their attachment to the tumor cells.

## Discussion

Most of the investigations on cytotoxicity effect of BCG in treatment of human bladder CIS, have been carried out in vivo (Takashi et al., 2000; Taniguchi et al., 1999). Different kinds of cell lines such as EJ137 and 5637 have been used to evaluate *in vitro* the cytotoxicity effect of Mycobacterium bovis Callmet and Guerin(Aghaei et al., 2015; Hayashi et al., 2009; Zuiverloon et al., 2017).

Two types of the immortal cell lines; EJ137 and 5637 were used in this study as the target for evaluation of possible alteration in cytotoxixcity effect of BCG in the presence of some lyoprotectants including trehalose, lactose, dextran-40 and MSG.

The amido black assay has been already used by (Schulz ET AL., 1994) to measure cytotoxicity of some cell lines. Although MTT as a prevalent toxicity assay has been employed in many routine related tests and researches (Chen et al., 2005), we preferred amido black assay because of likely affecting of the results by interfering of metabolism of BCG bacilli.

There are assured evidence of the cytotoxicity ability of the mycobacterium bovis BCG against urothelial cell lines and some other cells (Yu et al., 2015; Yang et al., 2014) investigated the anticancer activity of Luteolin (Lu) and its synergism effect with bacillus calmette-guerin (BCG) on human bladder cancer cell line BIU-87. The findings of Fanghong Chen, 2005 (Chen et al., 2005) showed that BCG exerts a direct cytostatic effect on human urothelial carcinoma cell lines.

Carbohydrates such as trehalose, lactose, dextran, and some amino acids such as mono sodium glutamate (MSG) are been used extremely as the stabilizer excipients of drugs and vaccines and as lyoprotectant in lyophilized products including interavesical BCG (Sefidi et al., 2017) although usually safety data of the proposed exposure to the excipients may be needed before marketing. Lactose is well recognized as a safe pharmaceutical excipient for use in vaccines, oral or inhalation formulations and is not likely to constitute any significant toxicological hazard (Baldrick & Bamford, 1997).

Trehalose, was considered as a non or less toxic and highly efficient lyoprotective agent for different kinds of cells or organisms[1] (Chen et al., 2016).

The data fond by (Lee et al., 2013) showed that trehalose at the concentration of 8mg/ml was noncytotoxic in their experiments.

Dextran-40 which has been investigated largely to intent using as cell lyoprotectant known as a safe additive in vaccine formula too (Sefidi et al., 2017).

Monosodium glutamate also has been known as a safe additive in foods and drugs. It is used to protect live and attenuated particles in bioproducts for a long term (Sefidi et al., 2017).

Since the desired excipients didn't reduce or increase the cytotoxicity effect of BCG, it could be concluded that they are nontoxic at the intended concentrations. According to our achievement, cytotoxicity of the BCG has not been affected by the presence of the substances even in 10% concentration. This means viability of BCG bacilli, attachment and penetration to the cells were not affected by these lyoprotectants. This finding indicated the safety of them in lyophilized intravesical BCG.

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