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Original Article

An Investigation into Improvement of Stability and Efficacy of Intravesical BCG Formulations Using Freeze-Drying Technique

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HIGHLIGHTS

- Lyophilization technique and lactose monohydrate as a protecting sugar were used to improve the stability, storage conditions, shelf-life and effcicay of intravesical BCG product.
- Maximum survival rate was attained for both liquid and lyophilized lactose-based formulations prepared with 10 %w/v lactose monohydrate and stored at less than -10 and 2-8°C, respectively.
- Freeze-dried products possessed higher stability and efficacy in comparison with corresponding liquid preparations.

ABSTRACT

Bacillus Calmette–Guérin (BCG) has been used as an intravesical product for the treatment of intermediate and high risk, non-muscle-invasive bladder cancer (NMIBC). Freeze drying technique is highly recommended for product development, however, the microorganism sensitivity to freezing and drying processes is a major chalenge which may lead to poor survival. To overcome this problem, the use of cryoprotectants in intravesical BCG formulation is required. This study was, therefore, planned to design a new formulation, using an attenuated strain of *Mycobacterium bovis*, which could be produced by freeze drying technique with the aim of prolonging its storage stability and increasing its efficacy as well as the ease of administration. For this purpose, sodium L-glutamate monohydrate (a commonly used stabilizer in domestic BCG suspension formulations) was replaced by lactose monohydrate. New intravesical BCG formulations, both in lyophilized and liquid forms, were eventually evaluated by moisture content assay, viable count assay, bacterial and fungal contamination, safety test and determination of bacterial concentration and O2 consumption. The results were compared with the data obtained for the conventional lyophilized and liquid products. Maximum survival rate was achieved in the presence of 10 % w/v lactose monohydrate for both liquid and lyophilized formulations when stored at less than -10 and 2-8°C, respectively. In summary, the freeze-dried formulations developed with lactose monohydrate met the requirements of intravesical BCG in high viability and stability during storage.

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Introduction

The most common type of bladder cancer is urothelial cell carcinoma which occurs in the cells lining the inside of the bladder. Depending upon the extent of cancer

spreading into the various layers of the bladder wall, bladder cancers can be described as non-invasive and invasive cancers. The former occurs only in the inner layer of cells (i.e., transitional epithelium), whereas in the latter, deeper layers are involved. However, a bladder cancer can also be described as non-muscle-invasive or superficial. Non-muscle-invasive bladder cancer (NMIBC) is found in the tissue that lines the inner surface of the bladder. This terminology implies that the tumor has not invaded into the true muscle layer in bladder. NMIBC, as a chronic disease with varying oncologic outcomes, is the most frequent malignancy of the urinary bladder and its high recurrence rate (up to 80%) and risk of progression (up to 30%) reflect the need for a long-term follow up and repeated treatments and sometimes multiple interventions (Jokisch et al., 2015). Surgery (transurethral resection of bladder tumor; TURBT), intravesical chemotherapy and immunotherapy have been considered as the main treatments for NMIBC.

Bacillus Calmette-Guérin (BCG), is a weakened (attenuated) strain of *Mycobacterium bovis* which is closely related to the one responsible for tuberculosis (i.e., *Mycobacterium tuberculosis*), but does not cause any disease in healthy people. BCG, which was primarily used as prevention of tuberculosis, is currently the only FDA-approved immunotherapy drug for the treatment of early-stage of bladder carcinoma. It has been reported that not only the frequency of recurrence is reduced by intravesical BCG, but also the tumor progression in patients suffering from high-grade NMIBC is significantly delayed, leading to increased survival rate (Sylvester et al., 2002; Jokisch et al., 2015).

Although it has been reported that the exact mechanism of intravesical BCG in the treatment of bladder cancer is not yet fully known, however, it is well recognized that BCG mainly influences the immune system. Once instilled locally, BCG bacteria contact the cancer cells and cause inflammation of bladder wall which in turn activates the immune system cells, through which the tumor cells are recognized and killed by cell-mediated mechanisms and for this reason, BCG therapy is considered an immunotherapy procedure. In addition, evidence show that BCG infection can also produce direct effects on tumor cells, and therefore, it is believed that antitumor acticity of BCG is a combination of direct effects and the immune system stimulation of the patient (Alexandroff et al., 1999; Gandhi et al., 2013; Kawai et al., 2013; Redelman-Sidi et al., 2014; Fuge et al., 2015).

Commercially available intravesical BCG product in Iran is manufactured by Pasteur Institute of Iran in the form of a single dose frozen suspension, with the final concentration of 40 mg/mL. This product is produced with an attenuated strain of *Mycobacterium bovis* (Pasteur strain 1173P2; the number of culturable particles is between 32-128 × 10^7/mL or 96-384 × 10^7/dose), contains sodium L-glutamate monohydrate (1.5 % w/v) and needs to be stored at the temperature of less than -10 °C. This product is labled for a shelf-life of 12 months and should be thawed and prepared before administration.

The stability of intravesical BCG when produced as a liquid preparation, is a major challenge. Freeze-thaw process of these liquid formulations has been found to increase bacterial dead mass which in turn reduces its efficacy. Therefore, it is worth developing a new form of this product that could be stored at milder storage conditions, while having adequate bacterial viability protection during its shelf life. This can be achieved by using freeze-drying technique in a stabilizing medium. Lyophilization is a preferred method which is usually used for the improvement of stability and shelf-life of biologicals that are unstable in aqueous medium. Traditionally, lyophilization cycle consists of three steps, namely freezing, primary and secondary drying. In the first step, cooling of the liquid formulation causes water ice crystals to form and grow, leaving a matrix of solutes. These ice crystals are removed through sublimation in the primary drying by lowering the pressure and supplying enough heat and finally the remaining unfrozen water molecules are desorbed by raising the temperature during the secondary drying step (Franks, 1998; Pikal et al., 2002; Liu et al., 2005; Kasper and Friess, 2011; Jin et al., 2011). However, inspite of the advantages offered by freeze-drying, many microorganisms may lose their activity during this process. It is necessary to ensure that product contains a high proportion of live bacilli with a desired stability on storage. In the case of intravesical BCG, sodium glutamate is conventionally used in the formulation as a stabilizer, but other protectants such as lactose can also added to the formulation in an attempt to avoid aggregation during lyophilization, depending upon the resistance of BCG strains (Surana et al., 2004; Kawai et al., 2005; Quaak et al., 2010; Jin et al., 2011).

In our previous study, we investigated the effect of trehalose as a cryoprotectant on the efficacy and stability of the intravesical BCG product in both liquid and freeze-dried forms (Hozouri et al., 2014). We found that trehalose could considerably increase the stability of the product against freezing and drying processes, increase the survival rate even in the liquid formulations, as well as the production of an acceptable cake. In the present study, we employed lactose monohydrate as a cryoprotectant (Hubálek, 2003) in various concentrations and therefore, developed a new BCG formulation. We also carefully selected both primary and secondary drying processing conditions to establish a reliable and efficient lyophilization process. It was

hypothesized that changing the stabilizer and using lyophilization technique would have probably considerable impacts on both the stability and efficacy of this product. The efficacy and stability of developed formulations, in both liquid and freeze-dried forms, were evaluated and compared with efficacy and stability of both liquid and lyophilized glutamate-based intravesical BCG products.

Materials and Methods

Materials

Live, attenuated strain of *Mycobacterium bovis* BCG (strain 1173P2), Lowenstein Jansen's medium and guinea pigs were gifted by Pasteur Institute of Iran. L-Asparagine monohydrate was obtained from Applichem Co. (Germany). Lactose, sodium glutamate, ferric ammonium citrate, magnesium sulfate $7H_2O$, dibasic and monobasic potassium hydrogen phosphate, dibasic sodium hydrogen phosphate, citric acid, ammonium 25%, zinc sulfate $7H_2O$, methanol and glycerin 87% were provided by Merck Co. (Germany). Trypton Soy Broth (TSB) and thioglycolate broth culture medium were purchased from Himedia Co. (India). Rubber stoppers were supplied by Helvoet Pharma Co. (Belgium). Aluminium caps and 10R glass vials were purchased from Sarsaz and Pars Ampoul Companies (Iran), respectively.

Preparation of concentrated Sauton

The concentrated Sauton was prepared as a nutrient culture medium for the growth of BCG. The ingredients of this culture medium, including L-asparagine monohydrate, zinc sulfate $7H_2O$, ferric ammonium citrate, magnesium sulfate $7H_2O$, potassium hydrogen phosphate, citric acid and glycerin 87% were added to purified water and the mixture was then autoclaved (Getinge GE, Sweden) at 121 °C for 20 minutes. The pH of the medium was eventually adjusted to 7.2 ± 0.1, using ammonium 25%.

Preparation of bulk solutions

Working seed lot was cultured on concentrated Sauton for 3 times. Incubation was performed at 37 ± 0.5°C (Memert, TV60b, Germany) for five weeks and the harvested cake was utilized for the preparation of concentrated bulk, following washing with phosphate buffer (pH = 7.38). Various amounts of lactose monohydrate (10, 15 and 20 % w/v) were used for the production of bulk solutions. Once prepared, these solutions were then transferred into 10R glass vials (3 mL in each vial) and stored in quarantine freezer at -20°C. In this study, the freeze-dried and liquid lactose-containing formulations were labeled as F-Lac and L-Lac preparations, respectively. Liquid (L-Glu) and lyophilized (F-Glu) intravesical BCG products, prepared domestically with 1.5%w/v sodium glutamate in Pasteur Institute of Iran, were used for comparative studies. All experiments were performed in triplicate.

Lyophilization protocol

A lyophilization protocol was designed and developed based on influencing factors such as moisture content and cake appearance and DSC data. The filled vials with half-seated stoppers were placed on the shelf of a freeze-dryer (Usifroid, SMH 50, France), already adjusted at 5°C. The shelf temperature was then reduced from 5°C to -50 °C at an appropriate rate. The vials were kept frozen at the temperature of -50°C for 3 hours. In the second stage, the temperature was increased to -33 °C at a specific rate and held constant for 6 hours. Finally, the secondary drying phase was performed by further increasing the temperature to 35 °C at a preset rate and pressure. The vials were stoppered and sealed by aluminum caps under vacuum and stored at 4 °C and 25°C (room temperature) for further characterizations and stability studies.

Cake appearance in lyophilized samples

Various sample vials were selected and their appearance was observed visually using an eye control device. The desired samples should be appeared in the form of lyophilized uniform white powder.

Moisture content assay (for lyophilized formulations)

The residual moisture of the lyophilized samples were evaluated through coulometric Karl-Fischer titration (Mettler Toledo , DL37 , Switzerland). To extract water, anhydrous methanol (2 mL) was added to the vials of freeze-dried intravesical BCG formulations and 1 mL of methnolic solution was then transferred to Karl-Fischer device. Based on British Pharmacopoeia (BP, 2003) requirements, the acceptable moisture content should be less than 3%.

DSC (Differential Scanning Calorimetry)

Glass transition temperature (T_g) of lactose and sodium glutamate were determined using a calibrated differential scanning calorimeter (Mettler Toledo Netzsch, 200 F3, Switzerland). Pans with approximately 10 mg of sample were cooled from room temperature to -80 °C at a decreasing rate of 10 °C/min, held at -80 °C for 5 min and then heated from -80 °C to 40 °C with an increasing rate of 10 °C/min (Her and Nail, 1994).

Count of viable units (Colony Forming Unit test)

Samples, prepared by reconstituting 0.1 mg of each freeze-dried formulation with 3 mL Sauton medium, were placed in Lowenstien-Jansen's medium. The samples were then incubated at 37 ± 0.5 °C for five weeks until colony forming units (CFU) with visible size were developed. The number of viable units per mL was determined by viable counting technique (BP, 2012).The survival rate for the lyophilized formulations was calculated by comparing the CFU values obtained immediately after the freeze drying process with those obtained after 6 months storage at room temperature (25°C) and 12 months at 4°C. Similarly, the same procedure was applied for the liquid suspensions and the survival rate was also determined through comparison of the viability values at the time of sample preparation with those obtained after 6 months storage at 4 °C and 12 months at -20 °C. In order to evaluate the effect of lyophilization process on the survival rate, the viability values before and immediately after the freeze drying were also calculated and compared for lyophilized formulations.

Bacterial and fungal contamination

All formulations were cultured on Tryptone Soy Broth (TSB) and Thioglycollate Broth (TGB). TSB and TGB media were incubated at 20-25 °C (for the detection of fungi), and were incubated at 30-35 °C (for the isolation of bacteria). All media were continuously evaluated for two weeks (WHO, 2013).

Determination of bacterial concentration via optical density

Total bacterial concentration can be indirectly determined by an opacity method (optical density; OD), calibrated in relation to the mass of the microorganisms. One mL of intravesical BCG with the concentration of 40 mg/mL was transferred to the test tube and 9 mL sodium glutamate or lactose monohydrate solution (with various concentrations) was then added to the tube and shaken until a uniform mixture was achieved. Similarly, this stock solution was diluted to the concentration of 0.5 and 1 mg/mL, using sodium glutamate and lactose monohydrate solutions. In accordance with WHO guideline (WHO, 2013) and British Pharmacopoeia (BP, 2012), bacterial concentration was determined by measuring OD at the wavelength of 490 nm.

Safety test (virulent mycobacteria)

Five mg of each formulation (liquid or freeze-dried) were injected subcutaneously into 6 guinea-pigs, each weighted between 250-400 g with no antibiotic treatment. These animals were closely monitored for at least 6 weeks. Minor reactions at the site of injection was ignored. If the animals remained healthy, gained weight and showed no signs of progressive tuberculosis within 6 weeks, the injected formulation was considered to be free from virulent mycobacterium (WHO, 2013; BP. 2012). At the end of this period, the guinea-pigs were also examined by autopsy for the signs of infection with tuberculosis.

Stability test

The stability of all freeze-dried and liquid formulations was analyzed at different storage temperatures, according to ICH Q5C (Guidance provided by International Conference of Harmonization, 2011). In general, accelerated stability tests were executed for 6 months at a temperature lower than 30 °C (room temperature) for freeze-dried samples and at 4 °C for liquid preparations. Long term stability tests were conducted for 12 month at 4 °C and -20 °C for freeze-dried and liquid preparations, respectively. Survival rate and optical density measurements, safety and microbial contamination tests were carried out for both freeze dried and liquid preparations, whereas the moisture content assay and the evaluation of the cake appearance were only conducted for the freeze-dried formulations. To determine the survival rate in freeze- dried samples, CFU values (as the main and effective quality parameter) obtained immediately after the freeze drying process were compared with those obtained for samples stored 6 months storage at room temperature (25 °C) or 12 months at 4 °C. This parameter was also determined for liquid formulations by comparison of the viability values at the time of preparation with those obtained after 6 months storage at 4 °C and 12 months at -20 °C. In an attempt to evaluate the influence of lyophilization process on the survival rate, the viability values in lyophilized vials were also determined and compared before and immediately after the freeze drying process.

Determination of O_2 consumption (Warburg test)

Warburg test is a method to measure CO_2 production and O_2 consumption based on the amount of oxygen utilized in the microorganism biologic oxidation. This test was carried out using 3 mL of the product and the amount of oxygen taken up by the microorganism was measured from time to time by considering the change in gas volume.

Statistical analysis

Data were compared statistically using GraphPad Prism version 8.0.1 (GraphPad Software, Inc., USA). Difference were assumed to be significant at $p < 0.05$.

Results and Discussion

Intravesical BCG product is domestically produced by Pastuer Institute of Iran with live, attenuated Pasteur strain 1173P2 of BCG and marketed in the form of frozen suspension vials. This product needs to be stored at less than -10 °C and thawed and prepared before administration. The mean shelf-life of the product at this condition is labeled 12 months after manufacturing. During freeze-thaw process,the bacterial survival rate decreases (i.e., an increase in the dead mass), leading to the reduction of the product efficacy. To enhance the stability and prolong the shelf-life of the intravesical BCG formulation well as its efficacy, and increase the possibility of storage in milder conditions (2-8 °C), the use of freeze-drying technique was considered. Although lyophilization technique is generally preferred for biological development, however, bacterial sensitivity to freezing and drying processes is a major challenge which may cause poor survival rate (Grant et al., 2009, Jin et al., 2011, Kasper and Friess, 2011). To overcome this problem, addition of some excipients to the formulation in order to stabilize them during freeze drying process and their storage are necessary. In this comparative investigation, it was decided to use lactose monohydrate with different concentrations as the stabilizer and protectant excipient in the formulation of intravesical BCG and the efficacy and stability of these lactose-based products, in both liquid and freeze-dried forms, were evaluated through various tests. The obtained results were compared with those obtained for both liquid suspension and lyophilized dosage forms of the existing immune BCG product, containing sodium glutamate.

Suitable lyophilized formulations should ensure adequate survival rate of microorganism as well as quick and complete reconstitution, long shelf-life, low residual moisture, elegant cake appearance and efficient biologic activity upon reconstitution. In fact, it is necessary to maintain the freeze temperature below glass transition temperature (T_g) of product to minimize damage during primary drying. In this regard, DSC is a thermal analysis method which is utilized for the optimization of lyophilization protocol (Her and Nail, 1994). T_g can be estimated by DSC and determined by the composition of the protectant and the residual moisture content (Hatley, 1992). In this investigation, DSC was employed for the development of lyophilization protocol. Based on the Tg values of lactose and sodium glutamate (Hozouri, 2014), the freeze drying protocol was established, as described earlier. Fig. 1 depicts the cake appearance of lyophilized BCG formulations prepared with 10 % w/v lactose monohydrate and 1.5 % w/v sodium glutamate.

(a) *(b)*

Figure 1. Cake appearance of freeze-dried BCG formulations prepared with a) 10% w/v lactose monohydrate and, b) 1.5% w/v sodium glutamate.

Viable count (survival rate)

Survival rate of the bacteria upon lyophilization and storage is one of the crucial quality attributes of freeze-dried formulations. Thus, the number of colonies cultured on Lowenstein Jansen's medium, before and after freeze drying and upon storage at different conditions was counted. Primary results showed that the survival rates of both liquid and freeze-dried formulations made up with 15 and 20 % w/v lactose monohydrate were not statistically different from those prepared with 1.5 % w/v sodium glutamate. Therefore, we compared the results of 10% w/v lactose monohydrate wth those obtained from sodium glutamate 1.5%. Data in Table 1 shows the viability values of freeze-dried BCG formulations containing either 1.5% w/v sodium glutamate or 10% w/v lactose monohydrate before and after lyophilization and the corresponding survival rates following freeze drying and 12 months storage at 4 °C.

As indicated in Table 1, the survival rates of bacilli immediately after freeze drying were noticeably higher in formulations prepared with lactose than those which contained sodium glutamate. In addition, long-term stability data showed that the mean survival rate was significantly higher in lactose-based lyophilized formulations in comparison with sodium glutamate-based preparations (p <0.05). This can be attributed to enhanced preservation effect of lactose over the other ingredient. These results clearly show better protective effect of lactose on bacteria during the process of freezing and drying than sodium glutamate and after a long term storage. The same trend was observed for the

viability values and the corresponding survival rates of liquid intravesical BCG formulations containing 1.5% w/v sodium glutamate and 10% w/v lactose at the time of sample preparation and after 12 months storage at -20 °C (Table 2). The superior survival rates of bacteria in liquid formulations also showed that lactose could act as an effective protector for bacteria during storage time in freezing condition compared to sodium glutamate.

Residual moisture

The residual moisture assay was performed for all lyophilized products at the time of preparation and within the storage period at various temperature conditions. Since the sublimation process in the lyophilization technique is an important stage which could influence the quality of the freeze-dried powder, if the residual moisture content of the product remains in the acceptable limit during the storage time, this could be an indication of an appropriate lyophilization protocol. In this study, it was found that the residual moisture content of all freeze dried formulations was less than 3% (in accordance with pharmacopoeial requirements for freeze-dried BCG vaccine) during the storage for 6 months at room temperature and 12 months at 2-8 °C, irrespective of the type and concentration of the protectant exicipients. However, results showed that the residual moisture content was relatively lower in formulations prepared with lactose monohydrate compared with sodium glutamate-based formulations. On the other hand, it was found that an increase in the lactose concentration from 10 to 20 % w/v had no effect on the moisture content. By considering these results, it was concluded that the lyophilization protocol selected in this project was an effective protocol for the intravesical BCG products.

Bacterial and fungal contamination

All formulations were found to be free from other bacteria, fungi and virulent mycobacteria. This indicated that there was no intervention with stability evaluation and survival rate determination.

Bacterial concentration

Bacterial concentration, an indicative of bacterial mass, was determined spectrophotometrically via optical density (OD) method which is a convenient and most widely used technique for the determination of the growth state of a bacterial cell culture. In this method, it is assumed that the obtained OD value is proportional to the sample concentration, however, a calibration technique is necessary for successful estimation of the density of cells in a liquid culture from OD measurement. As mentioned earlier, in this study, OD

Table 1. The viability values for freeze-dried intravesical BCG formulations containing 1.5% w/v sodium glutamate and 10% w/v lactose monohydrate, before and after lyophilization and the corresponding survival rates following freeze drying and 12 months storage at 4 °C.

Formulation	Viability value before lyophilization ($\times 10^6$ CFU/mL)	Viability value after lyophilization ($\times 10^6$ CFU/mL)	Survival rate (%) after lyophilization	Survival rate (%) after 12 month
F-Glu-01	16.5	0.4	2.42	12.5
F-Glu-02	15.8	0.35	2.21	-
F-Glu-03	12.2	0.10	0.82	-
Mean ± SD (n=3)	14.8 ± 2.3	0.28 ± 0.16	1.82 ± 0.9	-
F-Lac-01	19.2	1.2	6.25	45.8
F-Lac-02	17.4	1.2	6.9	41.6
F-Lac-03	15	1.1	7.3	40.9
Mean ± SD (n=3)	17.2 ± 2.1	1.16 ± 0.05	6.82 ± 0.53	42.8 ± 2.6

Table 2. The viability values for liquid intravesical BCG formulations containing 1.5% w/v sodium glutamate and 10% w/v lactose at the time of sample preparation and after 12 months storage at -20 °C and the corresponding survival rates.

	Viability value at time zero ($\times 10^6$ CFU/mL)	Viability value after 12 months ($\times 10^6$ CFU/mL)	Survival rate (%) after 12 month
L-Glu-01	16.5	1.42	8.60
L-Glu-02	15.8	1.54	9.70
L-Glu-03	12.2	1.07	8.70
Mean ± SD (n=3)	14.8 ± 2.3	1.34 ± 0.24	9.0 ± 0.6
L-Lac-01	19.2	3/34	17.4
L-Lac-02	17.4	3.93	22.60
L-Lac-03	15	3.72	18.10
Mean ± SD (n=3)	17.2 ± 2.1	3.66 ± 0.3	19.4 ± 2.80

measurements were carried out at the wavelength of 490 nm. The following generalizations could be made about the results obtained:

- For all lyophilized formulations, OD substantially decreased over long term storage at 2-8 °C which might be attributed to the breakage of entangled bacterial structures. It was observed that the extent of OD reduction in the presence of 1.5 % w/v sodium glutamate was greater than that in lactose containing formulations, such that OD value was even lower than the acceptable range (0.33-0.53). This could be considered as the advantage of applying lactose monohydrate over sodium glutamate in the formulations.
- Liquid formulations with 15 and 20 % w/v lactose monohydrate demonstrated more decrease in OD values, compared with samples with lower content of the protecting sugar (10 %w/v), over the long term storage at -20°C.
- In the case of freeze-dried samples stored at room temperature for 6 months, maximum reduction in OD values was observed in formulations composed of sodium glutamate and higher concentrations of lactose monohydrate.
- The same trend in OD decay was observed for liquid forms which were stored at 2-8 °C for 6 months, in accelerated stability test. Maximum decay occurred in samples prepared with sodium glutamate and higher concentrations of lactose monohydrate.

Determination of O_2 consumption (Warburg test)

Due to a good correlation between the amount of oxygen consumed by BCG bacilli and the live and active mass count in the formulation, it was decided to perform Warburg test as a rapid test for the evaluation of stability and efficacy of formulations prepared with different sugars and stored at various temperature conditions for 3 months . Fig .2 and Fig. 3 depict the diagrams of Warburg test results at the time of sample preparation and after 1, 2 and 3 months storage in refrigretaor (2-8 °C) and freezer (-10 to -25 °C) in the presence of sodium glutamate (1.5 %w/v) and lactose monohydrate (10 %w/v), respectively and Fig. 4 compares the corresponding survival rates. In general, it was concluded that freeze-drying technique and the use of lactose monohydrate as the cryoprotectant could increase the survival rate, provided that the formulation is kept at 2-8 °C. Although among the freeze-dried formulations that were stored in room temperature for 3 months, the percentage of survival rate was also higher in the presence of 10 %w/v lactose monohydrate, however, due to the significant decrease in the survival rate, it was concluded that the room temperature condition was not, in general, suitable for the storage of BCG formulations, even though lyophilized.

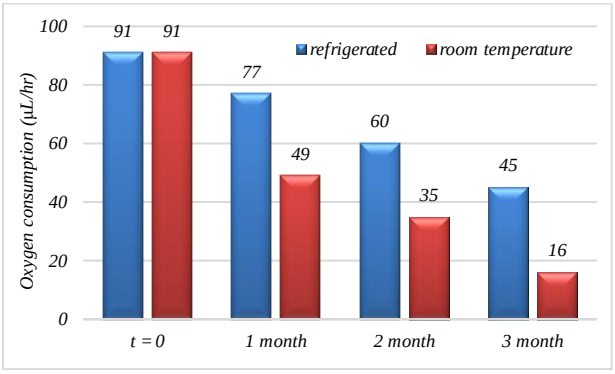

Figure 2. Warburg test results (oxygen consumption in terms of µL/hr) obtained for lyophilized BCG formulations prepared with 1.5 %w/v sodium glutamate, after 3 months storage at various temperatures.

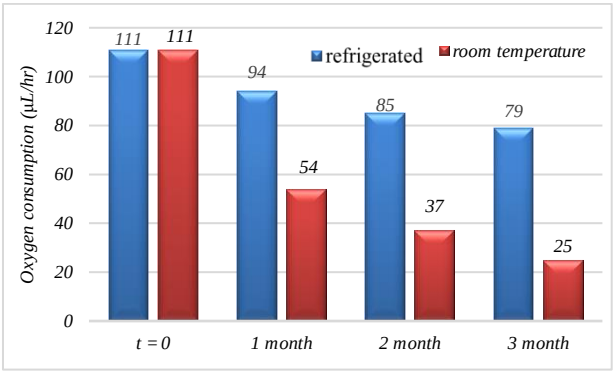

Figure 3. Warburg test results (oxygen consumption in terms of µL/hr) obtained for lyophilized BCG formulations prepared with 10 %w/v lactose monohydrate, after 3 months storage at various temperatures.

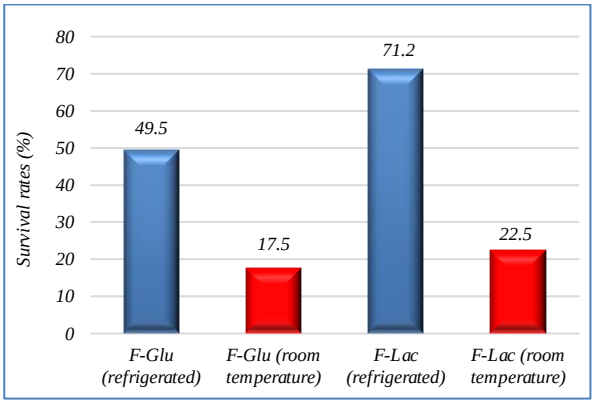

Figure 4. Corresponding survival rates (%) for lyophilized BCG formulations prepared with 10 %w/v lactose monohydrate or 1.5 %w/v sodium glutamate, after 3 months storage at various temperatures.

Conclusion

The goal of this study was to develop a better formulation for the intravesical BCG product. The use of lactose monohydrate as the stabilizer in the formulation and the application of freeze-drying cycle not only resulted in the production of an elegant cake, but also improved the storage conditions, viability and survival rates. These improved features could ensure that patients suffering from bladder cancer would probably receive adequate treatment doses of immune BCG, as well as potentially allowing transportation in milder storage conditions and ease of reconstitution and administration. Although our results seem to be promising, however, further experimental and clinical research is also required.

Ethical Statement

All procedures of the study have been performed on the basis of standard operating procedures and confirmed by the provisions of Declaration of Helsinki.

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Competing Interests

The authors declare that there is no conflict of interest.

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Authors' Contribution

All authors have made substantial contributions to the experimental design of this project. Experimental procedures and data collection were carried out by H. Hozouri. Analysis and interpretation of data were performed by all authors. This manuscript was initially prepared by H. Hozouri and revised by R. Aboofazeli before submission and all authors approved the final manuscript for submission This study was a part of Ph.D. thesis of H. Hozouri, proposed and approved by the School of Pharmacy, Shahid Beheshti University of Medical Sciences, Iran.

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