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CHARACTERIZATION, CELL PROLIFERATION AND CYTOTOXICITY EVALUATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR LOADED POLY (LACTIC-CO-GLYCOLIC ACID) MICROSPHERES

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ABSTRACT

Objective: *The aim of this study was to encapsulate vascular endothelial growth factor (VEGF) in poly(lactic-co-glycolic acid) (PLGA) microspheres using a water-in-oil-in-water emulsification method. Particle size distribution and surface morphology of PLGA microspheres and VEGF loaded PLGA microspheres were investigated. The effect of VEGF in free form and VEGF loaded in PLGA microspheres were also evaluated in the cell culture for cell proliferation and cytotoxicity.*

Material and Method: *Particles were sized by laser diffractometry. In vitro release profiles of VEGF from the microspheres were investigated in pH 7.4 phosphate buffer. The VEGF release was assessed using the enzyme linked immunosorbant assay (ELISA). The surface morphology of the microspheres was determined by a scanning electron microscobe. For the evaluation of the cytotoxicity of the formulations and proliferation effect of VEGF, the tetrazolium dye assay (MTT test) was performed.*

Results: *The microspheres were found to be spherical with particle size ranges of 8-12 µm for PLGA microspheres and 8-159 µm for VEGF loaded PLGA microspheres and the in vitro release results indicated that VEGF was released from the microspheres up to 30 days. According to the cell culture results, the formulations were non-cytotoxic and helps cells proliferate.*

Conclusion: *VEGF loaded microspheres were successfully prepared and their physical properties and in vitro release rate and cytotoxicity tests showed that the microspheres could be used for further in vivo experiments regarding nerve graft prefabrication.*

Key Words: *VEGF, PLGA, microspheres, controlled release, cell proliferation, cytotoxicity* **Nobel Med 2012; 8(1): 77-82**

VASKULAR ENDOTELYAL BÜYÜME FAKTÖRÜ YÜKLÜ POLİ(LAKTİK-KO-GLİKOLİK ASİT) MİKROKÜRELERİNİN KARAKTERİZASYONU, HÜCRE PROLİFERASYONU VE SİTOTOKSİK OLARAK DEĞERLENDİRİLMESİ

ÖZET

Amaç: Bu çalışmada vaskular endotelyal büyüme faktörünün (VEGF), poli(laktik-ko-glikolik asit) (PLGA) mikrokürelerine su/yağ/su emülsifikasyon metodu kullanılarak yüklenmesi amaçlanmıştır. PLGA mikrokürelerinin ve VEGF yüklü PLGA mikrokürelerinin partikül boyut dağılımı ve yüzey morfolojisi incelenmiştir. VEGF ve VEGF yüklü PLGA mikrokürelerinin hücre proliferasyonu ve sitotoksisitesi hücre kültüründe değerlendirilmiştir.

Materyal ve Metod: Mikrokürelerin boyutları lazer difraktometri ile belirlendi. Mikrokürelerin yüzey morfolojisi taramalı elektron mikroskobu ile incelendi. VEGF'in mikrokürelerden pH 7,4 fosfat tamponundaki in vitro salım profili enzyme linked immunosorbent assay (ELISA) kullanılarak incelendi. Formülasyonların sitotoksisitesi ve VEGF'in proliferasyon etkisini değerlendirmek için tetrazolyum boya tayini (MTT testi) kullanıldı.

Bulgular: PLGA mikrokürelerinin boyutları 8-12 µm, VEGF yüklü PLGA mikrokürelerinin boyutları ise 8-159 µm olup, küresel bir şekil gösterdiler. In vitro salım sonuçları mikrokürelerden VEGF salımının 30 gün boyunca devam ettiğini gösterdi. Hücre kültürü değerlendirmelerine göre, formülasyonların sitotoksik olmadığı ve hücre proliferasyonu yaptığı belirlendi.

Sonuç: VEGF yüklü mikroküreler başarılı bir şekilde hazırlandı. Fiziksel özellikleri, in vitro salım hızı ve sitotoksisite testleri mikrokürelerin sinir greft prefabrikasyonu ile ilgili in vivo çalışmalarda kullanılabileceğini gösterdi.

Anahtar Kelimeler: VEGF, PLGA, mikroküre, kontrollü salım, hücre proliferasyonu, sitotoksisite **Nobel Med 2012; 8(1): 77-82**

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a critical organiser of vascular development due to its ability to regulate proliferation, migration, specialization and survival of endothelial cells.[1] VEGF also affects many other cell types in tissue culture models. It is mitogenic for lymphocytes, retinal pigment epithelium and Schwann cells.[2] It also stimulates the migration of haematopoietic precursors, monocytes/macrophages, neurons and vascular smooth muscle cells, and it promotes the survival of developing and mature neurons, as well as chondrocytes.[3-5] VEGF is best known for its angiogenic properties. It promotes the growth of new blood vessels during embryonic development, and it is also important in adult stage by supplying adequate amount of oxygen and nutrients to most tissues.[6] VEGF use in the treatment of peripheral and myocardial vascular disease has been reported, but short in vivo half life (~30 min) of VEGF and as well as its adverse effects on hemodynamics, including hypotension, tachycardia, reductions in cardiac output and stroke volume when given by bolus injection failed to show efficacy in clinical trials when compared with intravenous (IV) infusion.[7,8] Recent research indicates that VEGF monotherapy can be successful in angiogenic induction but control over delivery rate and total dose are essential for production of normally functioning vascular networks.[8] Poly(laktik-ko-glikolik asit) (PLGA) and its derivatives have been used in various controlled release (CR) applications such as nano/microparticles, microspheres, implants and scaffolds, due to their advantages that include extended release rates up to days, weeks or months, in addition to their biocompatibility and biodegradability.[9-14] Among those studies angiogenic response was generated by VEGF encapsulated PLGA microspheres.[11] PLGA undergo hydrolysis upon implantation into the body, forming biologically compatible and metabolizable moieties (lactic acid and glycolic acid) that are eventually removed from the body by the citric acid cycle. Polymer degradation products are formed at a very slow rate and hence they do not affect the normal cell function. These polymers have been tested for toxicity and safety in animal studies extensively and are currently being used in humans for resorbable sutures, bone implants, screws, contraceptive implants, graft materials for artificial organs and as supporting scaffolds in tissue engineering research.[15-19] In this work, we aimed to develop formulations of VEGF loaded PLGA microspheres by using the method of water-in-oil-in-water emulsification, characterize the microspheres by particle size distribution, surface morphology, cell proliferation, cytotoxicity and subsequently investigate their in vitro release properties. Further work will be done to obtain nerve graft prefabrication by using VEGF loaded PLGA microspheres.

MATERIAL and METHOD

Materials: VEGF consisting of two 165 amino acid residue subunits with a molecular weight of approximately 39 kDa, PLGA of (50:50) lactic to glycolic acid copolymer ratio with a molecular weight of 40,000-75,000, albumin from rat serum (RSA) and polyvinyl alcohol (PVA) with a molecular weight of 72,000 and degree of hydrolysation 97.5-99.5 mol % were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Enyzme Linked Immunosorbent Assay (ELISA) kit was obtained from R&D Systems Inc., Minneapolis, MN, USA. All reagents used were of analytical grade.

Preparation of Microspheres: VEGF was encapsulated into PLGA microspheres using a water-in-oil-in-water emulsification method, following a protocol previously described (10), briefly VEGF (5 or 10 µg) and RSA (100 mg) and magnesium hydroxide (20 mg) were dispersed in 4 ml PLGA solution in methylene chloride (25% w/v) using a homogenizer (IKA, Labortechnik T 25 basic) at 8000 rpm for 1 min. A PVA solution (20 ml, 1 or 2% w/v) was added to this mixture and homogenized at 8000 rpm for a further 3 min. This emulsion was poured into 300 ml of an 0.1% (w/v) PVA solution and stirred for 1 h in a propeller mixer (IKA, Labortechnik RH basic) to evaporate the organic solvent. This procedure was conducted in an ice bath. The microspheres hardened were centrifuged (Hettich, Zentrifugen, Universal 32 R) at + 4ºC, 2650 g for 15 minutes and washed three times with distilled water then subsequently lyophylized (Christ, Alpha 1-2 LD Plus, Germany) for 24 hours.

Particle Size Distribution: Particles were sized by laser diffractometry using a Malvern 2000 laser sizer (Malvern Instruments Ltd., Malvern, UK). The average particle size was expressed as the volume mean diameter (v_{md}) in µm.

Surface morphology: The surface morphology of the microspheres was determined by a scanning electron microscobe (JEOL/JSM-6335F) after coating the microspheres with 10 nm palladium/gold on an aluminium stub.

In Vitro Release Study: VEGF loaded PLGA microspheres were placed in Eppendorf polypropylene tubes containing 1 ml of the release medium, phosphate buffer solution (PBS) (pH 7.4) and incubated at 37±0.5 ºC with constant agitation at 100 rpm in a shaking water bath (Certomat WR, B. Braun Biotech International). Five hundred microliters (µl) of PBS were periodically withdrawn from the tubes and replaced to maintain the VEGF concentration within sink conditions.→

At each time point (days 1, 3, 5, 7, 10, 13, 17, 21, 25, 30) the tubes were centrifuged and the supernatant was collected for analysis. The amount of VEGF released in the medium was measured using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. This quantitative assay is based on the classical sandwich enzyme immunoassay technique and involved incubation of 100 µl aliquots of the sample for 2 h at room temperature in a micro plate pre-coated with a monoclonal antibody specific for VEGF followed by the addition of a conjugate enzyme-linked polyclonal antibody for recognition of the immobilized VEGF and further incubation for 2 h. The substrate solution was then introduced to the microplates and the ezymatic reaction was allowed to progress for 30 min in dark that produced a colorimetric product whose intensity was proportional to the amount of VEGF bound in the first incubation step. Optical density was measured using a microplate reader (Biotek Instruments ELx800, USA) set at 450 nm and sample values were quantified using a standard curve. The in vitro release studies were conducted in triplicate and mean values and standard deviations were calculated.

Cell Proliferation and Cytotoxicity Evaluation: L929 cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 U/mL-50 µg/mL Penicillin/ Streptomycin at 37ºC in a humidified incubator (Sanyo MCO-18AIC, Japan) containing 5% CO_2. For the evaluation of the cytotoxicity of the formulations and proliferation effect of VEGF, the tetrazolium dye assay (MTT test) was performed. Cells were introduced into 96 well, flat-bottomed plates at $5x10^3$ cells/100 µl/ well and cultured overnight. Following day, medium was removed. VEGF loaded microspheres, blank microspheres and VEGF solutions in PBS were added onto each well and cultured at 37ºC in a humidified incubator containing 5% CO_2. After 24 hours of incubation 20 µl of MTT [3-(4,5 dimethylthiazol-2-yl)-25 (diphenyl tetrazolium bromide)] solution (1mg/ml) was added to each well and incubated for 4 hours more in incubator at 37ºC. 80 µl 23% SDS solution in 45% dimethyl formamid solution (pH 4.7) was added and incubated overnight. The following day each well was read at 570 nm with a microplate reader (VERSAmax, Molecular Devices Corporation CA, USA). The cells without samples were used as control group.

Statistical Analysis

The results were expressed as means ± standard deviations. Unpaired, two-tailed Student's t-tests were performed at each time point. The threshold for statistical significance was at $p<0.05$.

RESULTS

Preparation of Microspheres: The most commonly reported method for the manufacturing of protein loaded PLGA microspheres is the double emulsion technique.[20-23] Formulations of free PLGA microspheres and VEGF loaded PLGA microspheres prepared by water-in-oil-in-water double emulsion technique can be seen in Table 1. Acidic environment generated during the degradation of PLGA matrix due to the formation of acidic monomers and oligomers could inactivate growth factors. Therapeutic proteins can be protected from degradation due to acidic environment by including a buffering base such as magnesium hydroxide into the formulation.[24] Protein aggregation at the interface can be inhibited by addition of albumin to the aqueous phase before emulsification. Albumin protects the theurapeutic proteins by preferentially adsorbing to the interface.[25,26] According to the information of including a buffering base such as magnesium hydroxide into the formulation can protect the proteins from degradation (24) and also according to the information of addition of albumin to the aqueous phase before emulsification can inhibit protein aggregation at the interface (25,26), we have added MgOH and RSA into the aqueous phase of the formulations of both free PLGA microspheres and VEGF loaded PLGA microspheres. According to these reports we have added RSA and MgOH into the inner aqueous phase of the formulations of both free PLGA microspheres and VEGF loaded PLGA microspheres. Two different doses of VEGF (5-10 µg) and two different concentrations of PVA (1 and 2%) as an aqueous inner phase were used throughout the formulation development of the microspheres (Table1).

Particle Size Distribution: The mean particle size of free PLGA microspheres was found to be 12±1.5 µm and 8±1.0 µm for F1 and F2 formulations respectively (Figure 1). It is reported in literature that increasing the drug: PLGA ratio increases the microsphere size.[27] In our work, we also observed that loading VEGF into PLGA microspheres caused bigger particles. The mean particle size of VEGF loaded PLGA microspheres were found to be 111±8.7 µm, 111±14.5 µm, 8±1.0 µm and 159±21.5 µm for FV1, FV2, FV3 and FV4 formulations respectively (Figure 1). Bigger diameter of FV1 and FV2 formulations might be attributed to the high concentration of PVA used in the dispersed phase. Increasing the viscosity of the dispersed phase causes bigger diameter of microspheres.[28,29] The larger diameter of FV4 formulation compared to other formulations might be attributed to the high drug: PLGA ratio.[27]

Surface Morphology: Free PLGA microspheres were found to be spherical in shape and exhibited a smooth surface morphology (Figure 2 a,b). VEGF loaded PLGA

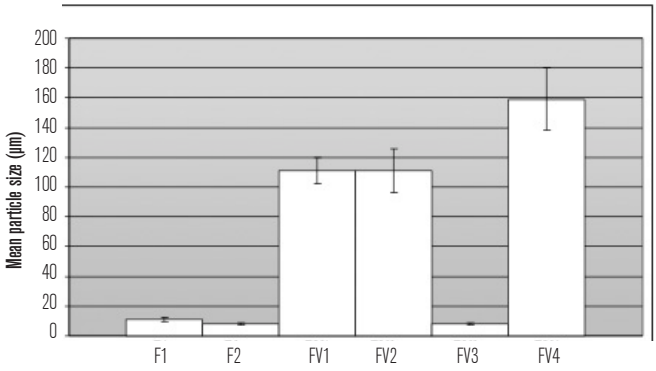

Figure 1. Size distribution of free PLGA microspheres prepared with a concentration of PVA 1% (w/v) as an aqueous inner phase (F1); free PLGA microspheres prepared with a concentration of PVA 2% (w/v) as an aqueous inner phase (F2); VEGF loaded PLGA microspheres prepared with 5 µg VEGF and 2% (w/v) concentration of PVA as an aqueous inner phase (FV1); VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 2% (w/v) concentration of PVA as an aqueous inner phase (FV2); VEGF loaded PLGA microspheres prepared with 5 µg VEGF and 1% (w/v) concentration of PVA as an aqueous inner phase (FV3); VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 1% (w/v) concentration of PVA as an aqueous inner phase (FV4). Data represents the mean ± S.D.

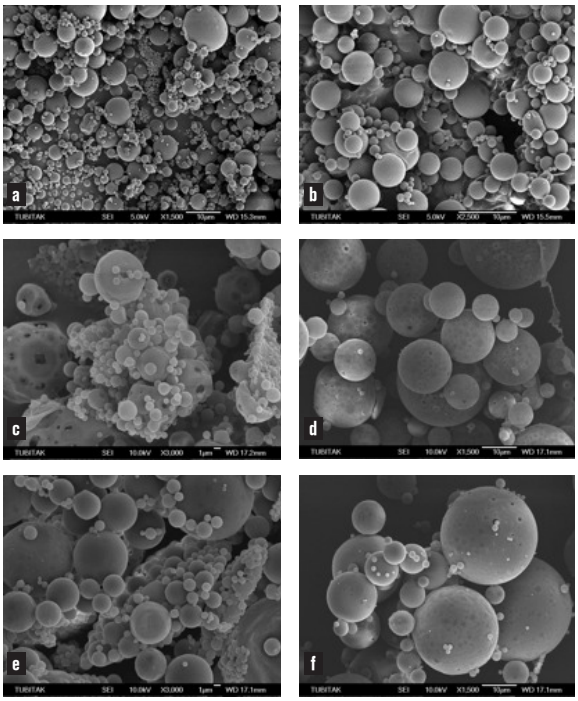

Figure 2. Scanning electron micrographs of a) free PLGA microspheres prepared with 1% (w/v) concentration of PVA as an aqueous inner phase (F1), b) free PLGA microspheres prepared with 2% (w/v) concentration of PVA as an aqueous inner phase (F2), c) VEGF loaded PLGA microspheres prepared with 5 µg VEGF and 2% (w/v) concentration of PVA as an aqueous inner phase (FV1), d) VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 2% (w/v) concentration of PVA as an aqueous inner phase (FV2), e) VEGF loaded PLGA microspheres prepared with 5 µg VEGF and 1% (w/v) concentration of PVA as an aqueous inner phase (FV3), f) VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 1% (w/v) concentration of PVA as an aqueous inner phase (FV4).

microspheres (FV1, FV2, FV3, FV4) were also spherical in shape but they exhibited porous surface morphology (Figure 2 c,d,e,f).[30] Increasing drug loadings of PLGA microspheres prepared by w/o/w solvent evaporation method resulted in more porous and more irregular shape.[31] It is clearly seen from our scanning electron microscopy photographs that loading VEGF to PLGA microspheres caused porous surface. Surface porosity regulates water inflow, outflow and drug release during incubation. It has also been reported in the literature that the formation of the surface pores originates from water mobility between inner and outer phases under osmotic pressure. Increasing osmotic potential increases pore size and number.[27,32,33]

In Vitro Release Study

In vitro release profiles of VEGF from VEGF loaded PLGA microspheres (FV2, FV4) are given in Figure 3. Controlled release of VEGF was achieved during 30 days period. At the end of 30 days 74.92±21.6% and 80.64±17.3% of VEGF were released from FV2 and FV4 microspheres respectively. Changing PVA concentration of the inner phase from 1% (w/v) to 2% (w/v) (Table 1) did not have a significant effect on the release profile of VEGF from VEGF loaded PLGA microspheres ($p>0.05$). The in vitro release rate data fit zero order kinetics between days 0 and 13 for FV2 and between days 0 and 10 for FV4 formulations (Figure 3).

The release of drugs from PLGA microspheres is considered to be due to diffusion of composite matrix along with simultaneous degradation of the PLGA polymer.[11,34] Degradation is a loss in the molecular weight of the PLGA chains and occurs via random hydrolysis, cutting polymeric chain into segments of various sizes and solubility.[11,33] Pore connectivity regulates drug diffusion and slower drug release may be the cause of low density of interconnecting channels.[11] Makino et al. showed pulsatile drug release in high molecular weight PLGAs. At lower molecular weight (19,000), a relatively constant release profile was obtained; increasing molecular weight to 23,000-44,000 and 74,000 decreased the linearity of release.[35] In our release data, an almost steady state release can be observed regarding the high molecular weight of PLGA used (40,000-75,000). Similar results with our study were reported by Kim, T. and Burgess, D. Their in vitro release data were zero order between days 10 and 30 with 65% of VEGF released at 30 days suggesting that release of VEGF from PLGA microspheres is controlled by the degradation of the polymer.[10] In another study, VEGF showed a burst release followed by a steady-state release from PLGA/PEG microspheres for at least 28 days.[9] Patil obtained a linear release of VEGF from PLGA microsphere/PVA hydrogel composites in vitro, showing approximately zero order kinetics up to 30 days.[11]

Cell Proliferation and Cytotoxicity Evaluation

In vitro cell culture studies have the advantage of relatively well-controlled variables and are generally accepted as a very effective method for biocompatibility testing; the sensitivity is equal or greater than that of in →

vivo studies.[36] The effects of VEGF solution with different concentrations, (1.25, 2.5 and 5 µg/ml), VEGF loaded in PLGA microspheres with different concentrations (0.25, 1.25 and 2.5 µg/ml) and control group on the viability of L-929 cell line were measured with MTT test. L-929 cells are being widely used in cytotoxicity analysis.[37-39] PBS was used as medium to remove the effect of DMEM during the incubation period. As it can be seen from Figure 4, as the concentration of VEGF and VEGF loaded in PLGA microspheres decreased, the absorbance values were also decreased. The absorbance value of the lowest VEGF concentration was even more than the absorbance value of the control group. This result means that VEGF both in free form and in microspheres are making cell proliferation. Both formulations are non-cytotoxic and they are making proliferation because of VEGF.[40]

CONCLUSION

In this study, VEGF loaded PLGA microspheres were prepared using water-in-oil-in-water emulsification method. Characterisation of the physical properties of the microspheres such as particle diameter, surface morphology and in vitro release data as well as cytotoxicity/proliferation was evaluated. The microspheres exhibited spherical shape with particle size range of 8-12 µm for PLGA microspheres and 8-159 µm for VEGF loaded PLGA. The ELISA results indicated a controlled release of VEGF from PLGA microsphere up to 30 days. According to cytotoxicity tests, VEGF in free form and VEGF loaded in PLGA microsphere are non-cytotoxic and they are causing proliferation because of VEGF. Finally, it can be concluded that the VEGF loaded microspheres were successfully prepared and their physical properties and in vitro release rate and cytotoxicity tests showed that the microspheres could be used for further in vivo experiments regarding nerve graft prefabrication.

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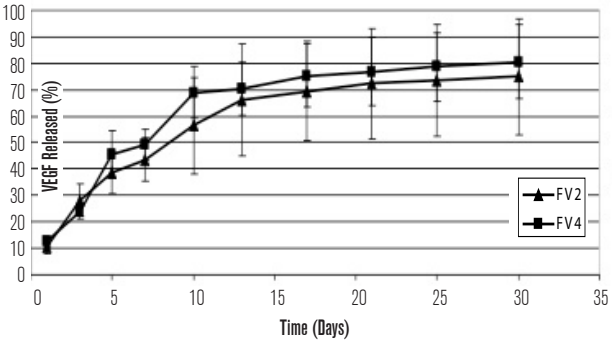

Figure 3. In vitro release profiles of VEGF from VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 2% (w/v) concentration of PVA as an aqueous inner phase (FV2) and VEGF loaded PLGA microspheres prepared with 10 µg VEGF and 1% (w/v) concentration of PVA as an aqueous inner phase (FV4) in pH 7.4 phosphate buffer solution for 1 month at 37°C. Data represents the mean±S.D. (n=3).

Table 1: Formulations of free PLGA microspheres and VEGF loaded PLGA microspheres prepared by water-in-oil-in-water double emulsion technique.

Formulation codes	VEGF (µg)	PLGA (g)	RSA (mg)	CH_2Cl_2 (ml)	1. PVA (ml)	MgOH (mg)	2. PVA (ml)
F1	-	0.5	50	4	10 (1%)	20	150 (0.1%)
F2	-	0.5	50	4	10 (2%)	20	150 (0.1%)
FV1	5	0.5	50	4	10 (2%)	20	150 (0.1%)
FV2	10	0.5	50	4	10 (2%)	20	150 (0.1%)
FV3	5	0.5	50	4	10 (1%)	20	150 (0.1%)
FV4	10	0.5	50	4	10 (1%)	20	150 (0.1%)

VEGF: vascular endothelial growth factor, PLGA: in poly (lactic-co-glycolic acid), RSA: rat serum, PVA: polyvinyl alcohol

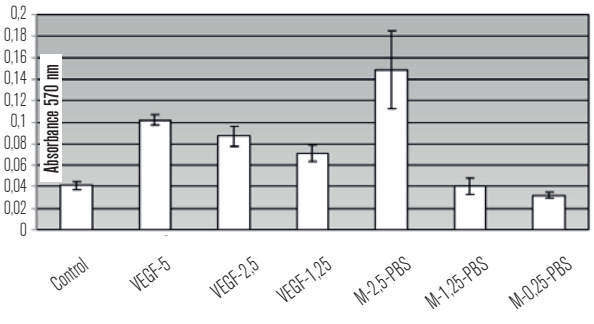

Figure 4. Cell proliferation and cytotoxicity evaluation of VEGF solution with different concentrations (1.25, 2.5 and 5 µg/ml), VEGF loaded in PLGA microspheres with different concentrations (0.25, 1.25 and 2.5 µg/ml) and control group on the viability of L929 cell line.

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