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Original Article

Preparation of Pluronic F127 hydrogel loaded with BIF1-iRGD recombinant protein for its targeted anti-cancer effects

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Abstract

Background and purpose: Pluronic F127-based hydrogel is a fair formulation for increasing the protein stability and half-life without decreasing its biological activity. The present study aimed to prepare FP127 hydrogel loaded with the recombinant BIF1-iRGD protein as a new immunotoxin with targeting potential of cancer cells.
Experimental approach: BIF1-iRGD in 19% w/v of FP127 was prepared by the cold method, and its *in vitro* release was determined. MTT and flow-cytometry assays were performed to evaluate the cytotoxic and apoptotic effects of BIF1-iRGD and BIF1-iRGD-hydrogel against 4T1 cells. The tumor size of 4T1 Balb-C mice was evaluated, and H&E staining was used for histopathology evaluation.
Findings/Results: BIF1-iRGD release followed the first-order model. The toxicity of BIF1-iRGD was less than that in the formulation as a hydrogel after 48 and 72 h of treatment. The null hydrogel showed no toxicity compared to the untreated cells. After 24 h, cells treated with BIF1-iRGD-hydrogel and BIF1-iRGD around their 48-h IC_{50} value developed apoptosis at about 55% and 35%, respectively. The tumor size decreased over time in the treated mice during the 20 days after the last injection. Also, no significant difference was observed between the effectiveness of the two groups. Tumor sections of mice treated with BIF1-iRGD and BIF1-iRGD-hydrogel had necrotic parts of about 65% and 70%, respectively.
Conclusion and implications: BIF-iRGD-hydrogel showed *in vivo* anticancer effects with increased toxicity in comparison to the native protein. However, the investigation of protein distribution and probable cytotoxic effect on vital organs must be noted.

Keywords: Apoptosis; Balb-C mice; BIF-iRGD; Hydrogel; Pluronic F127.

INTRODUCTION

In 2023, more than 300 thousand and 43 thousand new cases of breast cancer and its related deaths were recorded in the USA, respectively (1). For women, breast cancer accounts for 31% of female cancers (1). One of the new approaches in breast cancer treatment is the use of apoptosis-inducing molecules. An example in this group of proteins is BAX-interacting factor (BIF1). BIF1 is a 365-amino acid protein that plays an important role in apoptosis, autophagy, and the morphology of mitochondria (2). It has been shown that the defect in this protein leads to the inhibition of apoptosis and tumor induction (3). Furthermore, the expression level of BIF1 decreases in different cancers, such as colon (2,4), gastrointestinal tract (5), prostate (6), and breast (7) cancers, and this decrement is related to the poor prognosis and increase in cancer metastasis.

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So, a logical approach to compensate for the decreased level of this apoptotic protein in breast cancer is its exogenous replacement. We designed a new fusion protein named BIF1-internalizing arginine-glycine-aspartic acid (iRGD) and produced it by recombinant DNA technology. This protein contained iRGD as the targeting moiety that was recognized by the αvβ3 integrin receptors overexpressed on the TNBC cell line, MDA-MB-231 (8). The cytotoxic effects of this protein were also surveyed on MCF-7 cells (with moderate expression of αvβ3 receptors) and compared with the native protein produced in a similar way (BIF-1) (9). In the present project, the anti-cancer effects of the mentioned bio-molecule were evaluated *in vitro* and *in vivo*.

However, there are several challenges for *in vivo* protein delivery because of their intrinsic properties, such as fragile structures, short *in vivo* half-lives, and in some rare cases, potentially high toxicity (10). Thus, great efforts have been made over the past few decades in the development of polymeric delivery systems, particularly hydrogels, for protein delivery (11).

Hydrogels are hydrophilic polymer-based systems that are frequently used in drug delivery because of their noticeable properties, such as biocompatibility, easy encapsulation of proteins, and the ability to adjust their cross-linked network and composition, which controls the release of entrapped proteins, protects proteins against proteolytic degradation, and finally, prolongs their bioactivity (12). Thermosensitive hydrogels are they with the ability to increase their viscosity with increased temperature, as well as simpler application and longer durability time at the site of application (13). Poloxamer or Pluronic® F127 (FP127) is a copolymer with the ability to form a thermosensitive hydrogel with low toxicity and favorable rheological properties, as well as its solubility (14). There are several studies in which various proteins have been loaded in poloxamer hydrogels and used for *in vitro* and *in vivo* projects (15,16). One study revealed that hirudin has been developed as a thermosensitive gel using FP127 to increase its anti-thrombotic effects as well as its bioavailability without any drug-polymer interactions and change in stability (15). In another study, IL-1Ra was loaded in FP127, resulting in a significant increase in serum half-life and also led to its sustained release (16). Furthermore, FP127 hydrogel was used for the subcutaneous administration of insulin, and it was shown that this formulation prolonged the hypoglycemic effects of insulin (17).

Taken together, it seems that FP127-based hydrogel is a fair formulation for increasing the protein stability and its half-life without adverse effects and decreasing its biological activity. So, the aim of the present study was to prepare FP127 hydrogel loaded with the recombinant BIF1-iRGD fusion protein, followed by *in vitro* and *in vivo* evaluation on 4T1-bearing Balb-C mice.

MATERIALS AND METHODS

Plasmids, chemicals, and cell lines

The recombinant pTWIN1 vector containing BIF1-iRGD was prepared by Biomatik Company (Canada). *E. coli* BL21 (DE3) and 4T1 cell line were prepared from Pasteur Institute (Iran) and used as the recombinant production host and the cell with over-expression of αvβ3 receptors on the cell surface, respectively. FP127, ampicillin, and other chemicals were purchased from Sigma (Germany), and finally, cell culture consumables and equipment were prepared from BioIdea (Tehran, Iran).

BIF1-iRGD expression and purification

The *E. coli* BL21 (DE3) was transformed by recombinant pTWIN1-BIF1-iRGD and cultured in Luria Bertani medium containing 100 µg/mL of ampicillin at 37 °C for 16 h in a rotatory shaker, and then about 1% of this culture was added to 1 L of fresh medium containing ampicillin until reaching the logarithmic phase determined by spectrophotometry. In the next stage, the induction of soluble expression of the BIF1-iRGD proteins was done by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) in a final concentration of 0.15 mM for 16 h at 15 °C. Then, the bacteria were extracted from the culture media *via* centrifuging at 7000 × *g* for 10 min at 4 °C, and the protein expression was investigated by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

IMPACT™ purification system was used for the cleavage induction of intein1 fused to the N-terminal of BIF1-iRGD protein. For sample preparation, about 25 mL of B1 buffer (Tris-HCl 20 mM, NaCl 500 mM, and EDTA 1 mM, pH 8.5)

was added to the 1 L of pellet of culture medium, and cell disruption was done by a sonication of 30 s 3 times and the sonication of 15 s 3 times with 2-min-intervals between sonication runs. After centrifugation at 7000 × *g* for 30 min at 4 °C, the supernatant was injected into the chromatography column containing 5 mL of chitin resin, which was previously equilibrated by the addition of B1 buffer. Then, the resin column was subjected to rotation on a rotary for 30 min. Washing the column was performed using B1 buffer for 5 times, and ultimately, 20 mL of B2 buffer (Tris-HCl 20 mM, NaCl 500 mM, EDTA 1 mM, 0.3% Triton X-100, and 0.2% Tween 20, pH 6.5) was added to the column and kept at room temperature for 24 h to induce the self-cleavage ability of intein 1. The eluted samples were evaluated on the following day by 12% SDS-PAGE.

The recombinant proteins were subjected to the lipopolysaccharide removal using Triton-X114 method, dialyzed against phosphate-buffered saline (PBS, pH 7.4) using a cellulose dialysis membrane 3 times, and finally, the protein concentration was determined using the Bradford method against various concentrations of human serum albumin (HSA) for drawing the standard curve, and serial dilution was performed by PBS (18,19).

Preparation of the temperature-sensitive FP127 hydrogel containing BIF1-iRGD

BIF1-iRGD-loaded FP127 hydrogel was prepared by the cold method. For this end, 1.90 g of FP127 powder and 1.5 mL of BIF1-Irgd (500 µg/mL) were dissolved in 10 mL of deionized water under stirring in an ice bath to obtain a clear solution containing 19% w/v of FP127 and 0.015% w/v of BIF1-iRGD. To determine the gelation temperature of the solution, 1 mL of formulation was put into a vial, immersed in a water bath at 15 °C, and then the samples were heated at a rate of 0.4 °C/min to the temperature at which gel formed. The vial was taken out every 1 min and inverted horizontally to examine the flowability of the samples. The temperature at which the solution lost its fluidity was taken as the gelation temperature. The null hydrogel was prepared as described above without the addition of BIF1-iRGD.

Drug release study

In vitro release of BIF1-iRGD from FP127 hydrogel was determined using a membrane-less method (16). Exactly, 2 mL of BIF1-iRGD-loaded hydrogel (500 µg/mL) and blank hydrogel were placed in a 10 mL beaker at 500 rpm and heated in a water bath at 37 °C until a clear gel was obtained. About 7 mL of PBS (37 °C, pH 7.4) was placed over the surface of the hydrogel. Then, beakers were put in a shaker (KBLee, Korea) at 100 rpm and 37 °C. At predetermined times, 200 µL of media was removed and replaced with the same volume of media. The amount of released BIF1-iRGD in the sample was determined by Bradford assay using HSA as a standard (20). For this purpose, the samples were concentrated 4 times and then mixed with the Bradford reagent based on a 1:10 (v/v) ratio in a 96-well plate; then they were incubated at room temperature for 15 min. The absorbance was measured at 595 nm on an ELISA reader (BioTek Instruments, Inc., 140213C, USA).

Release kinetic study

To describe the release kinetics, *in vitro* release data of BIF1-iRGD from FP127 hydrogel were fitted by the kinetic models, including zero order, first order, Higuchi, and Korsmeyer-Peppas using equations (1), (2), (3), and (4), respectively.

$$\frac{Q_t}{Q_\infty} = kt \pm b \quad (1)$$

$$Ln\left(1 - \frac{Q_t}{Q_\infty}\right) = -\mathrm{kt} \quad (2)$$

$$\frac{Q_t}{Q_\infty} = kt^{\frac{1}{2}} \quad (3)$$

$$\frac{Q_t}{Q_\infty} = kt^n \quad (4)$$

where k is the rate constant, Q_t/Q_∞ is the fraction of drug released at time (t), and n is the release exponent and determines the release mechanism (21).

Cell culture and treatment

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to evaluate the cytotoxicity of BIF1-iRGD and BIF1-iRGD-hydrogel against the mouse breast cancer cell line, 4T1. FP127 hydrogel without the fusion protein was also used as the negative control. About 20 µL of various concentrations (31.25, 62.5, 125,

250, and 500 µg/mL) of BIF1-iRGD or hydrogel containing equal amounts of the fusion protein was poured into the wells of a 96-well plate. Then, cell suspension in RPMI1640 supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), with the final density of 2×10^4 cells/mL, was seeded in each well in the final volume of 180 µL and incubated in a humidified 37 °C incubator with 5% CO_2 for 24 h. Four wells were assumed as a negative control, and the cells included were treated with free hydrogel. At the end of 48- or 72-h incubation, the cells were treated with 20 µL of MTT (5 mg/mL). After 3 h additional incubation, the content of the wells was discarded and formazan crystals were dissolved in 150 µL of dimethyl sulfoxide (DMSO). Finally, the plates were subjected to read absorbance at 570 nm by a microplate reader (Bio-Rad, USA).

Apoptosis assessment by flow cytometry

Based on cell viability assessment, the IC50 for BIF1-iRGD or BIF1-iRGD-hydrogel was calculated, and the cells were treated with the IC50 of the two surveyed groups. For this aim, Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (eBioscience™, USA) was used according to the manufacturer's protocol. Briefly, BIF1-iRGD and hydrogel containing BIF1-iRGD were added to wells of a 6-well plate as duplicates, and then the 4T1 cells with the final density of 5×10^5 cells/mL were seeded to all wells and incubated for 24 h in standard conditions. Then, the cells were collected and washed by PBS and binding buffer (1X), prepared by the Company, and incubated with Annexin-V-FITC and propidium iodide (PI) according to the manufacturer's instructions for 30 min in the dark. Finally, the cells were centrifuged at $300 \times g$ and washed using binding buffer (1X), suspended in 200 µL of binding buffer, and analyzed by flow cytometry on a BD FACSCalibur (BD, USA).

In vivo study

Twenty 4-week-old female BALB/c mice (22 ± 3 g) obtained from the animal house of the School of Pharmacy and Pharmaceutical Sciences (Isfahan, Iran) were used in this study. The animals were kept in standard conditions for temperature and humidity in the animal house of the School of Pharmacy and Pharmaceutical Sciences (Isfahan, Iran). All experimental procedures were performed according to the guidelines approved by the Ethical Committee of Isfahan University of Medical Sciences (Ethical Code: IR.MUI.RESEARCH.REC.1399.594).

For tumor inducing, about 200 µL of the suspension with a final concentration of 2×10^6 cells/mL of 4T1 cells was subcutaneously injected into the upper right hind leg of mice. Then, all mice were analyzed for their tumor volume every other day to reach 500-550 mm^3 before the initiation of treatment. In the next stage, xenograph mice were randomly divided into 4 groups and treated with 200 µL of various samples, including normal saline, native BIF1-iRGD (2 mg/kg), BIF1-iRGD-hydrogel, and native hydrogel by intratumoral injection every other day with a total of 5 doses. To determine antitumor efficacy, tumor growth and terminal tumor weight were evaluated. Tumor volume at specified intervals was calculated using equation (5).

$$Tumor\ volume = \frac{(W \times L_2)}{2} \quad \textbf{(5)}$$

where, W and L represent the widest diameter and the longest dimension, respectively. The animals were euthanized by CO_2 10 days after the last injection. After removing the tumor, tumor mass was calculated. Hematoxylin and eosin (H&E) staining was used to evaluate tumor histopathology.

Statistical analysis

For cell toxicity evaluation, each experiment was assessed in triplicate. PBS-treated cells were assumed as the negative control. The data were expressed as mean ± SD, and SPSS (Version 25) was used to compare the treatments by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The confidence interval for considering significant results was at least 95%. The IC_{50} of each recombinant protein was determined by drawing the graph of cell survival percent against concentration using GraphPad Prism 7.0 software. Finally, the comparison of the tumor volume and weight was performed by an independent T-test.

RESULTS

BIF1-iRGD expression and purification

Figure 1 represents the expression of BIF1-iRGD in fusion to intein 1 of the pTWIN1 vector and auto-cleavage of intein 1 from the BIF1-iRGD recombinant protein. According to the band related to the pellets of *E. coli* BL21 (DE3) transformed by the recombinant pTWIN1-BIF1-iRGD at 15 °C with 0.15 mM final concentration of IPTG, a band approximately in 70 kDa was shown, while for the bacterial cells transformed with the non-recombinant pTWIN1, a band approximately in 45 kDa was appeared, equal to the intein 1 and 2 fusion protein. Expression and purification of cloned genes in the pTWIN-1 vector as a fusion to intein 1 were assessed by SDS-PAGE. On the other hand, the auto-cleavage of intein 1 from the target protein (BIF1-iRGD) after incubation of the chitin column at room temperature for 24 h revealed bands in approximately 47 kDa, establishing the successful purification of BIF1-iRGD.

Finally, according to the Bradford method, the final amounts of the purified protein were calculated as 3.23 mg from 1 L of bacterial culture.

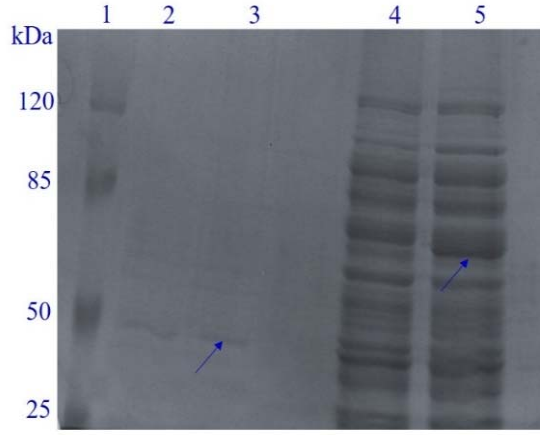

Fig. 1. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis analysis of BIF1-iRGD recombinant expression and purification. Lane 1, Protein marker; lanes 2 and 3, purified BIF1-iRGD after the digestion of intein 1; lane 4, uninduced *E. coli* BL21 (DE3) containing the recombinant pTWIN1 vector; lane 5, induced *E. coli* BL21 (DE3) containing the recombinant pTWIN1 vector by 0.15 mM isopropyl β-D-1-thiogalactopyranoside. BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid.

Hydrogel preparation and release assay results

BIF1-iRGD-loaded FP127 hydrogel showed temperature-dependent sol-gel phase transition. As shown in Fig. 2A-C, BIF1-iRGD-loaded FP127 hydrogel was a flowable liquid at room temperature and became a non-flowable gel at 37 °C. Figure 2D shows the release profile of BIF1-iRGD from FP127 hydrogel. To evaluate the kinetics of BIF1-iRGD release from the hydrogel, the experimental release data were fitted to different kinetic models, and the results are shown in Table 1. According to the obtained R^2 (correlation coefficient) values, BIF1-iRGD release followed the first-order model. In this case, the drug release rate was related to the concentration of the drug. In the Korsmeyer-Peppas plot, the obtained n value was about 0.5031, which explained the non-Fickian transport of BIF1-iRGD from the hydrogel. So, diffusion coupled with erosion controlled BIF1-iRGD release from the hydrogel.

Biological effects of BIF1-iRGD-hydrogel on 4T1 breast cancer cells

The MTT assay showed that cell survival was reduced in the cells treated with various samples (BIF1-iRGD and BIF1-iRGD hydrogel) in a concentration- and incubation time-dependent manner in comparison to the cells treated with PBS, assumed as the negative control. The results of the ANOVA test showed that the toxicity of native protein was less than that in the formulation as a hydrogel after 48-h treatment. On the other hand, the native hydrogel showed no toxicity in comparison to the untreated cells, which revealed that the more toxicity with BIF1-iRGD hydrogel can be attributed to the recombinant protein (Fig. 3A). Moreover, 72-h treatment of cells showed that the cytotoxicity of hydrogel containing the targeted protein was more than the native protein, which can be attributed to the more stability of the recombinant protein when formulated as hydrogel (Fig. 3B). Finally, according to the Fig. 3 (the survival percent versus concentration) for each sample, the IC_{50} of BIF1-iRGD and BIF1-iRGD in hydrogel was determined to be 11.9 and 5.1 µg/mL after 48-h incubation, respectively. Also, IC_{50} values of 24.3 and 12.8 µg/mL were reported for BIF1-iRGD and BIF1-iRGD in hydrogel after 72-h incubation, respectively.

Table 1. Correlation coefficient (R^2) obtained from curve fitting of BIF1-iRGD release data from FP127 hydrogel.

Formulation	Kinetic models (R^2)		Korsmeyer-Peppas parameters		
	Higuchi	First order	Zero order	n	R^2
BIF1-iRGD loaded FP127 hydrogel	0.9692	0.9885	0.9725	0.5031	0.9665

FP127, Pluronic F127; BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid.

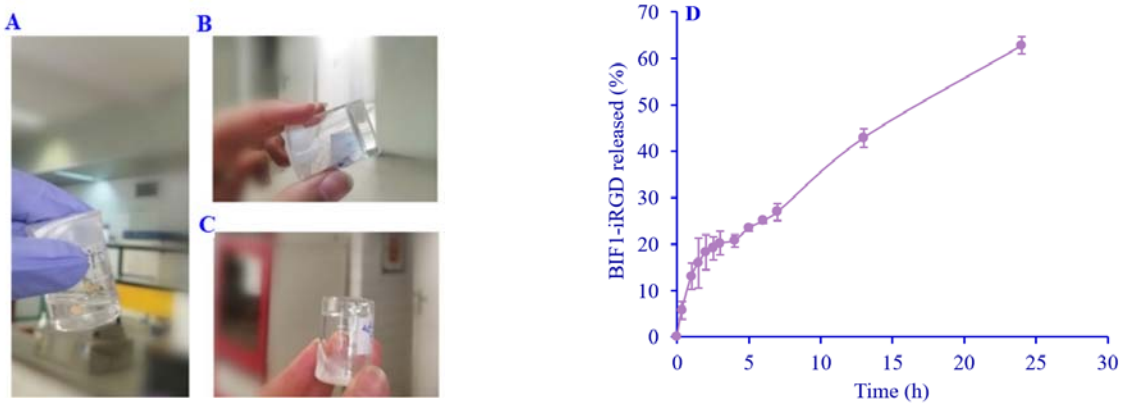

Fig. 2. (A-C) Thermosensitive FP127 hydrogel preparation at different temperatures; (A) room temperature; (B and C) 37 °C temperature; (D) release percent of BIF1-iRGD from FP127 Hydrogel over time. FP127, Pluronic F127; BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid.

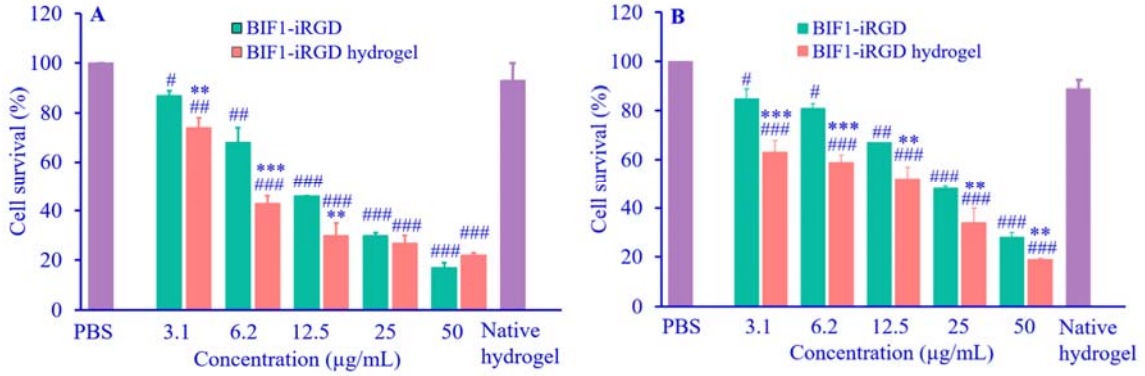

Fig. 3. Cytotoxicity evaluation of BIF1-iRGD-hydrogel in comparison to the native BIF1-iRGD after (A) 48 and (B) 72 h of incubation against 4T1 cells. Cells treated with PBS were considered the negative control. Data were expressed as mean ± SD (n =3) and analyzed by one-way ANOVA followed by Tukey post-hoc test. $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, and $^{\#\#\#}P \leq 0.001$ demonstrate significant differences compared to the PBS group; $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ versus BIF1-iRGD in the same concentrations. BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid; PBS, phosphate-buffered saline.

Evaluation of the apoptosis induction of BIF1-iRGD on 4T1 cells

The results of the flow cytometry assay showed that cells treated with the IC_{50} concentration of 48-h treatment (10 µg/mL for BIF1-iRGD and 5 µg/mL for BIF1-iRGD-hydrogel) developed apoptosis (early + late quadrant) at about 35% and 55% after 24-h incubation for BIF1-iRGD and BIF1-iRGD-hydrogel treated cells, respectively. Although this value for untreated cells was shown as about 23%. The survival percent of the untreated and cells treated with BIF1-iRGD in the hydrogel was about 77% and 55 %, respectively. For cells treated with BIF1-iRGD-loaded hydrogel, the apoptotic effects were increased to about 45%, and cells in the late apoptosis were also increased (Fig. 4). Altogether, the data established the apoptosis induction ability of BIF1-iRGD-loaded hydrogel in the cells.

In vivo anti-cancer effects of BIF1-iRGD hydrogel

Figure 5A shows tumor size change during 20 days after the last injection. In the mice treated with normal saline, no decrease was observed, and the tumor size was significantly larger than before the injection. Tumor size decreased in the mice treated with BIF1-iRGD-loaded hydrogel and free BIF1-iRGD in comparison to the negative control group. Also, no significant difference was observed between the effectiveness of these groups (Fig. 5A). Finally, histopathological evaluation of tumor sections by H&E staining showed that there was a large number of alive cancer cells in the normal saline-treated mice. In fact, only 10% of cells in the negative control group became necrotic. Tumor sections of mice treated with BIF1-iRGD and BIF1-iRGD-hydrogel also showed necrotic tissue. These values were estimated as about 65% and 70% for BIF1-iRGD and BIF1-iRGD-hydrogel, respectively. Exact surveys showed no difference between these groups (Fig. 5B-E).

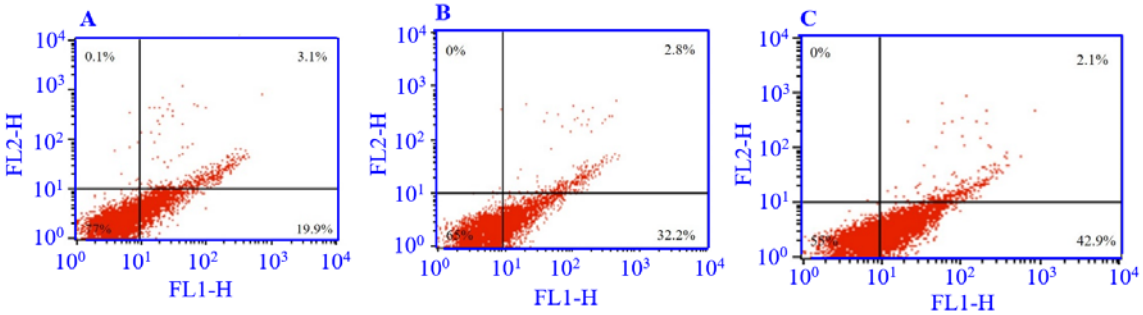

Fig. 4. Apoptosis evaluation of BIF1-iRGD-hydrogel and native BIF1-iRGD by flow cytometry after 24 h of incubation. (A) Untreated cells, (B) cells treated with BIF1-iRGD, (C) cells treated with BIF1-iRGD-hydrogel. Lower left chamber, live cells (annexin V^-/PI^-); lower right chamber, early apoptotic cells (annexin V^+/PI^-); upper left chamber, dead cells (annexin V^-/PI^+); upper right chamber, late apoptotic cells (annexin V^+/PI^+). BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid; PI, propidium iodide.

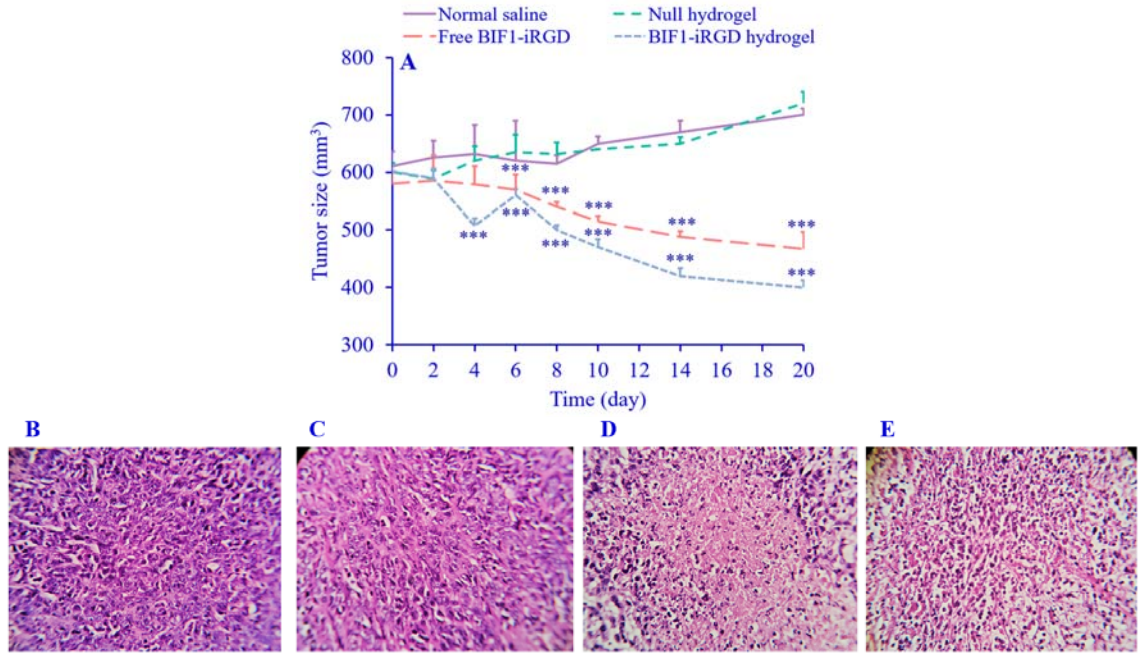

Fig. 5. (A) Tumor size change in various mouse groups over time. The normal saline group was considered the negative control. Data were expressed as mean ± SD, n = 5 and analyzed by an independent T-test. $^{***}P < 0.001$ shows a significant difference in comparison to the negative control at the same time. (B-E) H&E staining of the tumor tissues in the mouse groups treated with (B) normal saline; (C) null hydrogel; (D) native BIF1-iRGD; (E) BIF1-iRGD-hydrogel. BIF1, BAX interacting factor-1; iRGD, internalizing arginine-glycine-aspartic acid.

DISCUSSION

In the present study, the formulation of BIF1-iRGD as a FP127 hydrogel had no adverse effects on its performance in both conditions of *in vitro* and *in vivo*. The release profile of BIF1-iRGD from FP127 hydrogel showed that about 60% of the protein was released after 25-h incubation. The results of the MTT assay indicated that the most cytotoxic effects of BIF1-iRGD and hydrogel containing the targeted protein were observed after 48-h incubation. Actually, the effectiveness of BIF1-iRGD hydrogel was two-fold of the native fusion protein. After 72 h of incubation, although the cytotoxicity diminished for both groups, the effectiveness of the hydrogel containing the recombinant protein was more than that of BIF1-iRGD. BIF1-iRGD, which was designed and evaluated for its targeted cytotoxic effects in comparison to the native BIF1 recombinant protein, showed more toxicity against MDA-MB-231 breast cancer cells than MCF-7 cells in the previous study (9). Actually, there was no significant difference between the IC50 value of BIF1 against MCF-7 and MDA-MB-231, especially after 24 h of treatment.

There is no other evidence about the usage of BIF1 as a recombinant protein for its cytotoxic or apoptotic effects. In the present project, the percentage of apoptosis induction after 24 h of treatment was determined as 35% for the recombinant protein and 55% for the hydrogel loaded with BIF1-iRGD in their IC_{50} values. In our previous study, when the fusion protein was used at a final concentration of 0.65 µg/mL for 24 h, 69% of apoptosis was detected (data not published). The concentration used in the present study, with the same apoptotic effects, was about 10-fold greater than in our previous project, which can be attributed to the difference in the cell line.

In another study, Sun et al. showed that treatment of MDA-MB-231 cells with fluoxetine implicated BIF1 as a factor involved in apoptosis. They reported that fluoxetine could induce apoptosis in the cells by stimulating the expression of BIF1. Furthermore, the maximum expression of this protein occurred after 24 h, and the concentration of BIF1 decreased after 36 h of treatment with fluoxetine (22). Based on this finding, it seemed that the use of a formulation that leads to an increase in the durability of the protein at the site of action could help to increase its duration of action. As shown in the results section, the cytotoxic analysis confirmed that the intact FP127 hydrogel had no toxic effects in comparison to the negative control. On the other hand, it led to more toxicity of the fusion protein for both times of incubation.

The present study was the firstly to investigate the potential effects of BIF1-iRGD *in vivo* anti-cancer effects. The results confirmed that either native BIF1-iRGD or in hydrogel form caused cell necrosis and diminished the cancer cell population. In addition, several documents have examined the expression level of this apoptotic protein in various types of cancers (4-7).

In a study focusing on FP127 as a thermosensitive polymer for delivering proteins, a hydrogel composed of 2.7% (w/v) F127/PEG containing antibodies against T lymphocyte-associated protein-4 (CTLA)-4 and programmed cell death (PD)-1 was prepared and injected into tumors induced by 4T1 cell injection. The results showed that after 40 days of implantation of the hydrogel containing 50 µg of each monoclonal antibody, the greatest effect in reducing the tumor size occurred in comparison to the formulation with 30 µg of only one antibody or free antibodies in the same amounts. On the other hand, in the testing of *in vivo* hydrogel stability, after 16 days of injection, the size of the used hydrogel did not statistically decrease in comparison to its size on the first day (23). The authors of this study explained that thermosensitive and biocompatible F127-pSH containing aCTLA-4 and aPD-1 antibodies prolonged antibody release to afford superior tumor control with simultaneous reductions in systemic toxicity (23).

In another study, a 30% FP127 hydrogel was loaded with nanoparticles containing a pore-forming toxin (24). The retention of the mentioned nanoparticles by the FP127 hydrogel was surveyed *in vivo*, and it was established that in 48 h of the treatment of mice, the fluorescence intensity of the nude nanoparticle group showed a more rapid decay than that of the nanoparticles loaded in FP127. Quantitatively, more than 90% of the nanoparticles diffused over 48 h, while only approximately 40% of them were lost in the hydrogel-loaded group (24). Although the current study didn't detect the distribution of the fusion protein when injected in FP127 hydrogel in comparison to the native BIF1-iRGD, the *in vivo* anti-cancer effect investigation suggested that the preparation of this targeted protein with FP127 hydrogel has no interfering effect to reduce the anti-cancer effectiveness of BIF1-iRGD.

CONCLUSION

In the present study, a new formulation was prepared for a fusion protein (BIF1-iRGD) with cancer-targeted therapy potential and then surveyed *in vitro* cytotoxic and apoptotic effects of BIF1-iRGD as well as its *in vivo* anti-cancer effects when prepared in FP127 hydrogel. The present results showed that the null FP127 hydrogel had no toxic or anticancer effects. The toxicity percentage of the fusion protein increased when loaded into the hydrogel. BIF1-iRGD induced cell death via apoptosis. So, the active ingredient was effective with targeted ability. The selected formulation was also safe and led to more efficacy for the native protein. However, the investigation of protein distribution and probable cytotoxic effects on vital organs must be noted before beginning the clinical phases.

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Conflict of interest statement

All authors declared no conflict of interest in this study.

Authors' contributions

Z. Tamizifar performed the experiments and prepared the manuscript draft; A. Jafarian-Dehkordi conducted the animal tests; S. Taymouri conducted the preparation of the hydrogel and release test; F. Shafiee designed the study, conducted the fusion protein production, and molecular tests. All authors read and approved the final version of the manuscript. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

REFERENCES

1. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA Cancer J Clin. 2023;73(1):17-48. DOI: 10.3322/caac.21763.
2. Yoon H K, Young-Seok C, Hye Sung W, Ho Jung A, Der Sheng S, Soon Uk H, *et al*. Stage-stratified analysis of prognostic significance of bax-interacting factor-1 expression in resected colorectal cancer. Biomed Res Int. 2013;2013:1-8. DOI: 10.1155/2013/329839.
3. Takahashi Y, Karbowski M, Yamaguchi H, Kazi A, Wu J, Sebti SM, *et al*. Loss of BIF1 suppresses Bax/Bak conformational change and mitochondrial apoptosis. Mol Cell Biol. 2005;25(21):9369-9382. DOI: 10.1128/MCB.25.21.9369-9382.2005.
4. Pierrat B, Simonen M, Cueto M, Mestan J, Ferrigno P, Heim J. SH3GLB, a new endophilin-related protein family featuring an SH3 domain. Genomics. 2001;71(2):222-234. DOI: 10.1006/geno.2000.6378.
5. Gil1 J, Ramsey D, Szmida1 E, Leszczynski P, Pawlowski P, Bebenek M, *et al*. The BAX gene as a candidate for negative autophagy-related genes regulator on mRNA levels in colorectal cancer. Med Oncol. 2017;34(2): 16,1-7. DOI: 10.1007/s12032-016-0869-y.
6. Coppola D, Oliveri C, Sayegh Z, Boulware D, Takahashi Y, Pow-Sang J, *et al*. BAX-interacting factor-1 (BIF1) expression in prostate cancer. Clin Genitourin Cancer. 2008;6(2):117-121. DOI: 10.3816/CGC.2008.n.018.
7. Runkle KB, Meyerkord CL, Desai NV, Takahashi Y, Wang HG. BIF1 suppresses breast cancer cell migration by promoting EGFR endocytic degradation. Cancer Biol Ther. 2012;13(10):956-966. DOI: 10.4161/cbt.20951.
8. Naik A, Al-Yahyaee A, Abdullah N, Sam JE, Al-Zeheimi N, Yaish MW, *et al*. Neuropilin-1 promotes the oncogenic Tenascin-C/integrin β3 pathway and modulates chemoresistance in breast cancer cells. BMC Cancer. 2018;18(1):533,1-14. DOI: 10.1186/s12885-018-4446-y.
9. Ghobadi M, Shafiee F. Recombinant production of BIF1-iRGD fusion protein with cytotoxic and apoptotic effects against breast cancer cell lines. Biomed Res Ther. 2026. In press.
10. Lyu Y, Azevedo HS. Supramolecular hydrogels for protein delivery in tissue engineering. Molecules. 2021;26(4):873,1-31. DOI: 10.3390/molecules26040873.
11. Thang NH, Chien TB, Cuong DX. Polymer-based hydrogels applied in drug delivery: an overview. Gels. 2023;9(7):523,1-38. DOI: 10.3390/gels9070523.
12. Chai Q, Jiao Y, Yu X. Hydrogels for biomedical applications: their characteristics and the mechanisms behind them. Gels. 2017;3(1):6,1-15. DOI: 10.3390/gels3010006.
13. Fan R, Cheng Y, Wang R, Zhang T, Zhang H, Li J, Song S, *et al*. Thermosensitive hydrogels and advances in their application in disease therapy. Polymers (Basel). 2022;14(12):2379,1-21. DOI: 10.3390/polym14122379.
14. Cui N, Dai CY, Mao X, Lv X, Gu Y, Lee ES, *et al*. Poloxamer-based scaffolds for tissue engineering applications: a review. Gels. 2022;8(6):360,1-24. DOI: 10.3390/gels8060360.
15. Liu Y, Lu WL, Wang JC, Zhang X, Zhang H, Wang XQ, *et al*. Controlled delivery of recombinant hirudin based on thermo-sensitive Pluronic F127 hydrogel for subcutaneous administration: *in vitro* and *in vivo* characterization. J Control Release. 2007;117(3):387-395. DOI: 10.1016/j.jconrel.2006.11.024.

16. Hamid Akash MS, Rehman K, Li N, Gao JQ, Sun H, Chen S. Sustained delivery of IL-1Ra from pluronic F127-based thermosensitive gel prolongs its therapeutic potentials. Pharm Res. 2012;29(12):3475-3485. DOI: 10.1007/s11095-012-0843-0.
17. Nasir F, Iqbal Z, Khan A, Khan JA, Khan A, Khuda F, *et al*. Development and evaluation of pluronic- and methylcellulose-based thermoreversible drug delivery system for insulin. Drug Dev Ind Pharm. 2014;40(11):1503-1508. DOI: 10.3109/03639045.2013.831441.
18. Shafiee F, Rabbani M, Behdani M, Jahanian-Najafabadi A. Expression and purification of truncated diphtheria toxin, DT386, in *Escherichia coli*: an attempt for production of a new vaccine against diphtheria. Res Pharm Sci. 2016;11(5):428-434. DOI: 10.4103/1735-5362.192496.
19. Shafiee F, Yazdani A. Recombinant production of interleukin-1 receptor antagonist in fusion to albumin binding domain with potential affinity to human serum albumin. Res Pharm Sci. 2024;19(3): 356-365. DOI: 10.4103/RPS.RPS_41_23.
20. Severino P, Andreani T, Jäger A, Chaud MV, Santana MH, Silva AM, *et al*. Solid lipid nanoparticles for hydrophilic biotech drugs: optimization and cell viability studies (Caco-2 & HEPG-2 cell lines). Eur J Med Chem. 2014;81:28-34. DOI: 10.1016/j.ejmech.2014.04.084.
21. Karimi Z, Taymouri S, Minaiyan M, Mirian M. Evaluation of thermosensitive chitosan hydrogel containing gefitinib loaded cellulose acetate butyrate nanoparticles in a subcutaneous breast cancer model. Int J Pharm. 2022;624:122036. DOI: 10.1016/j.ijpharm.2022.122036.
22. Sun D, Zhu L, Zhao Y, Jiang Y, Chen L, Yu Y, *et al*. Fluoxetine induces autophagic cell death via eEF2K-AMPK-mTOR-ULK complex axis in triple negative breast cancer. Cell Prolif. 2018;51(2):e12402, 1-10. DOI: 10.1111/cpr.12402.
23. Kim J, Francis DM, Thomas SN. *In Situ* crosslinked hydrogel depot for sustained antibody release improves immune checkpoint blockade cancer immunotherapy. Nanomaterials (Basel). 2021;11(2):471,1-10. DOI: 10.3390/nano11020471.
24. Zou S, He Q, Wang Q, Wang B, Liu G, Zhang F, *et al*. Injectable nanosponge-loaded Pluronic F127 hydrogel for pore-forming toxin neutralization. Int J Nanomedicine. 2021;16:4239-4250. DOI: 10.2147/IJN.S315062.