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ORIGINAL ARTICLE

Antidiabetic exendin-4 activates apoptotic pathway and inhibits growth of breast cancer cells

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Abstract Exendin-4 is a GLP-1 analog used for the treatment of type 2 diabetes mellitus in its synthetic form. As women with diabetes have higher breast cancer incidence and mortality, we examined the effect of the incretin drug exendin-4 on breast cancer cells. The aim of the study is to investigate anticancer mechanism of exendin-4 in MCF-7 breast cancer cells. Cytotoxic effects of exendin-4 were determined by XTT assay. IC_{50} dose in MCF-7 cells were detected as 5 μM at 48th hour. Gene messenger RNA (mRNA) expressions were evaluated by real-time PCR. According to results, *caspase-9*, *Akt*, and *MMP2* expression was reduced in dose group cells, compared with the control group cells. *p53*, *caspase-3*, *caspase-8*, *caspase-10*, *BID*, *DR4*, *DR5*, *FADD*, *TRADD*, *PARP*, *PTEN*, *PUMA*, *NOXA*, *APAF*, *TIMP1*, and *TIMP2* expression was increased in dose group cells, compared with the control group cells. Effects of exendin-4 on cell invasion, colony formation, and cell migration were detected by Matrigel chamber, colony formation assay, and wound-healing assay, respectively. To conclude, it is thought that exendin-4 demonstrates anticarcinogenesis activity by effecting apoptosis, invasion, migration, and colony formation in MCF-7 cells. Exendin-4 may be a therapeutic agent for treatment of breast cancer as single or in combination with other agents. More detailed researches are required to define the pathways of GLP-1 effect on breast cancer cells because of the molecular biology of breast cancer that involves a complex network of interconnected signaling pathways that have role in cell growth, survival, and cell invasion.

Keywords Exendin-4 · Glucagon-like peptide · Breast cancer · Diabetes

Abbreviations

DM	Diabetes mellitus
GLP-1	Glucagon-like peptide-1
GPCR	G protein-coupled receptor
IGF-1	Insulin-like growth factor 1
XTT	2,3-Bis-(2-Metoksi-4-nitro-5-sülfofenil)-2H-tetrazolium-5-carboxanilide

Introduction

In addition to the known complications of diabetes, there is emerging evidence that diabetes mellitus (DM) patients may face a higher risk of developing cancer [1] and a higher cancer mortality [2], particularly for breast cancer [2, 3]. Various factors may be related to obesity, diabetes, and breast cancer risk, contain activation of insulin and insulin-like growth factor 1 (IGF-1) pathways, altered regulation of endogenous sex hormones, and altered levels of adipocytokines [4–9].

Glucagon-like peptide (GLP)-1 is situated in the incretin system and secreted from intestinal L cells. It demonstrates its actions via the GLP-1 receptor (GLP-1R)—a G protein-coupled receptor (GPCR) expressed on pancreatic β cells and other tissues, including the brain, heart, and smooth muscle [9, 10]. Different activities of GLP-1 have been defined to

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date. For instance, GLP-1 stimulates insulin secretion from β cells, modulates their proliferation and differentiation, and inhibits their apoptosis [9, 11]. Furthermore, GLP-1 is a strong inhibitor of gastric emptying and a promoter of satiety; sustained activation of the GLP-1R is related to weight loss [12]. Some researches suggest that GLP-1 may increase insulin sensitivity in peripheral tissues [13, 14]. Exendin-4 is a more stable GLP-1 analog [15] currently used for the treatment of type 2 diabetes mellitus in its synthetic form exenatide [16].

GLP-1-based therapy on breast cancer may have significant clinical implications because of the fact that type 2 DM, obesity, and breast cancer commonly appear together [2, 16]. Recently, several studies have been reported on the effects of GLP-1 and exendin-4 on cancer cells. In these studies, an inhibitory effect of exendin-4 on cell growth in colon CT26 [17] and in MCF-7 and MDA-MB-231 breast [9] cancer cells has been reported. We aimed to evaluate the effects of exenatide on breast cancer cell proliferation and viability; besides, this evaluates the effects on cell migration, cell invasion, and colony formation capacity in this study.

Material and methods

Cell culture

MCF-7 human breast cancer cell (obtained from ATCC, USA) line was used in this study. MCF-7 cells were grown in DMEM medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10 % (*v*/*v*) heat-inactivated fetal calf serum at 37 °C in a saturated humidity atmosphere containing 95 % air and 5 % CO_2. MCF-7 cells were treated with 0.25, 0.5, 1, 1.5, 2, 3, 5, 7.5, and 10 μM exendin-4 by solving in medium during 72 h, considering a time- and dose-dependent manner.

Cytotoxicity assay

Cytotoxicity assays and determination of IC_{50} dose of exendin-4 in MCF-7 cells were performed by using trypan blue dye exclusion test and XTT assay as indicated in the manufacturers' instruction.

XTT assay

Cells were seeded in 96-well tissue culture plates and incubated for 24 h without reagent. After addition of reagents, cells were incubated for 24, 48, and 72 h and cell viability was assessed by using XTT (2,3-bis-(2-Metoksi-4-nitro-5-sülfofenil)-2H-tetrazolium-5-carboxanilide) mixture as recommended by the supplier. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 630 nM) using a microplate reader. Viability was calculated using the background-corrected absorbance as follows:

$$\text{Viability}(\%) = \text{A of experiment well}/\text{A of control well} \times 100$$

RNA isolation and real-time PCR

Total RNA was isolated from the cells exposed to IC_{50} doses of exendin-4 with TRIzol Reagent (İnvitrogen, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis was performed by using Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany). *p53*, *caspase-3*, *caspase-9*, *caspase-8*, *caspase-10*, *Bcl-2*, *BCL-XL*, *BID*, *DR4*, *DR5*, *FADD*, *TRADD*, *PARP*, *APAF*, *Bax*, *Akt*, *PTEN*, *PUMA*, *NOXA*, *MMP2*, *MMP9*, *TIMP1*, *TIMP2* gene expression was performed on real-time RT-PCR according to the SYBR Green qPCR Master Mix (Thermo Scientific, USA) protocol. RT-PCR assay was performed using gene-specific primers. The expression results were proportioned to the *GAPDH* gene (housekeeping gene) expressions to calculate relative expression rations. Primer sequences were given in Table 1.

Cell migration and invasion assay

Invasion activities of control and dose group cells were determined according to the BioCoat Matrigel Invasion Chamber guide (BD Biosciences). The cells with serum-free DMEM were seeded at a density of 2×10^5 cells/well onto the upper chambers of Matrigel-coated filter inserts, and serum-containing DMEM (500 μl) was added to the lower chambers. Then, the cells were incubated at 37 °C for 24 h. After of incubation, filter inserts were removed from the wells. The cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with crystal violet. The cells that invaded the lower surface of the filter were counted under a light microscope. Each experiment was repeated three times. Percentage of invasion was calculated using control and Matrigel membrane cell count as follows:

$$\text{Invasion}(\%) = \frac{\text{The number of cells in matrigel matrix basement membrane}}{\text{The number of cells in control membrane}} \times 100$$

Colony assay

For colony formation analysis, the cells were digested with trypsin, counted using trypan blue dye exclusion test, and seeded in six-well plates at a density of 10^3 cells per well. The medium was changed every 3 days for 15 days until visible colonies formed. Colonies were fixed methanol for 10 min and stained with crystal violet.

Table 1 Primer sequences of the genes used in this study

Name	Primer sequence
APAF	F: ATGAGCCCACTCAACAGCAA
	R: GTCCTTACACTGGAAGAAGAGAC
BAX	F: AGAGGATGATTGCCGCCGT
	R: CAACCACCCTGGTCTTGGATC
Caspase-3	F: GCAGCAAACCTCAGGGAAAC
	R: TGTCGGCATACTGTTTCAGCA
BCL-2	F: TTGGCCCCCGTTGCTT
	R: CGGTTATCGTACCCCGTTCTC
GAPDH	F: TTCTATAAATTGAGCCCGCAGCC
	R: CCGTTGACTCCGACCTTCAC
Caspase-9	F: GGCTGTCTACGGCACAGATGGA
	R: CTGGCTCGGGGTTACTGCCAG
p21	F: TGGAGACTCTCAGGGTCGAAA
	R: GGCGTTGGAGTGGTAGAAATC
p53	F: ATCTACAAGCAGTCACAGCACAT
	R: GTGGTACAGTCAGAGCCAACC
PTEN	F: CCCAGACATGACAGCCATC
	R: TCTGCAGGAAATCCCATAGC
PARP	F: ACACCCCTTGCACGTACTTC
	R: GATGGGTTCTCTGAGCTTCG
PUMA	F: GACCTCAACGCACAGTACGAG
	R: AGGAGTCCCATGATGAGATTGT
NOXA	F: ACCAAGCCGGATTTGCGATT
	R: ACTTGCACTTGTTCCTCGTGG
BID	F: CCTACCCTAGAGACATGGAGAAG
	R: TTTCTGGCTAAGCTCCTCACG
AKT	F: CAACTTCTCTGTGGCGCAGTG
	R: GACAGGTGGAAGAACAGCTCG
BCL-XL	F: GGTCGCATTGTGGCCTTTTTC
	R: TGCTGCATTGTTCCCATAGAG
DR4 (*TNFRSF10A*)	F: GCGGGGAGGATTGAACCAC
	R: CGACGACAAACTTGAAGGTCTT
DR5 (*TNFRSF10B*)	F: ACAGTTGCAGCCGTAGTCTTG
	R: CCAGGTCGTTGTGAGCTTCT
FADD	F: GCTGGCTCGTCAGCTCAAA
	R: ACTGTTGCGTTCTCCTTCTCT
TRADD	F: GCTGTTTGAGTTGCATCCTAGC
	R: CCGCACTTCAGATTTCGCA
Caspase-8	F: TCTGGAGCATCTGCTGTCTG
	R: CCTGCCTGGTGTCTGAAGTT
Caspase-10	F: TAGGATTGGTCCCCAACAAGA
	R: GAGAAACCCTTTGTCGGGTGG
MMP2	F: TCTCCTGACATTGACCTTGGC
	R: CAAGGTGCTGGCTGAGTAGATC
MMP9	F: CCTTGTGCTCTTCCCTGGAG
	R: GGCCCCAGAGATTTCGACTC
TIMP1	F: ACCATGGCCCCCTTTGAGCCCCTG
	R: TCAGGCTATCTGGGACCGCAGGGA
TIMP2	F: CTCGGCAGTGTGTGGGGTC
	R: CGAGAAACTCCTGCTTGGGG

Wound-healing assay

The control and dose group cells were plated at 10^6 cells per well of 60×15 mm style cell culture dishes and grown overnight at 37 °C with 5 % CO_2. The 80 % confluent control group and dose group cells were treated with 5 μM exendin-4 after a straight line scratch was made on a confluent monolayer of cells using a sterile 200-μl plastic pipette tip. To remove debris and smooth the edge of the scratch, the cells were washed with 2 ml serum-free DMEM. Images of the MCF-7 cell proliferation were taken at 0 16, 24, and 48 h after the scratch. The scratch assay was performed in triplicate.

Statistical analysis

The analysis of the findings has been made with the ΔΔCT method and quantitated with a computer program. The comparison of the groups has been performed with "Volcano.Plot" analysis, from "RT^2.ProfilesTMPCR Array Data Analysis", which is assessed statistically using the "Student *t* test." Moreover, parametric and non-parametric analysis of doses and controls has been evaluated with the SPSS 17.0 statistical analysis program ($p<0.05$ is significant statistically).

Results

Cytotoxic activity

XTT assay

MCF-7 cell death upon treatment with exendin-4 was assessed by the XTT (2,3-bis-(2-Metoksi-4-nitro-5-sülfofenil)-2H-tetrazolium-5-carboxanilide) assay. Time- and dose-dependent decrease patterns were found in the viability of MCF-7 cells. For this purpose, the expression changes of genes are evaluated by treating different concentration of exendin-4 at the 24th, 48th, and 72nd hour in MCF-7 cells. In our study, IC_{50} doses (inhibitory concentration where 50 % of the cells die) in the MCF-7 cells were detected as 5 μM at 48th hour by XTT assay (Fig. 1).

Real-time PCR

After total RNA was isolated from control and exendin-4-treated cells, the cDNA synthesis have been performed by using Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics, Germany). The expression analysis of *p53*, *caspase-3*, *caspase-9*, *caspase-8*, *caspase-10*, *Bcl-2*, *BCL-XL*, *BID*, *DR4*, *DR5*, *FADD*, *TRADD*, *PARP*, *APAF*, *Bax*, *Akt*, *PTEN*, *PUMA*, *NOXA*, *MMP2*, *MMP9*, *TIMP1*, *TIMP2* was studied on real-time RT-PCR according to the SYBR Green qPCR Master Mix (Thermo Scientific) protocol. Real-

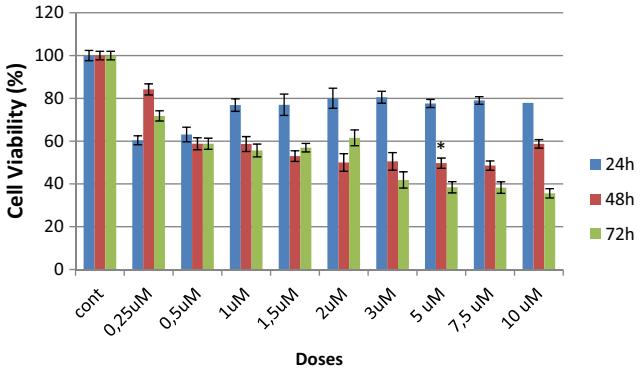

Fig. 1 Effect of exendin-4 on the viability of MCF-7 cells. The cells were treated with exendin-4 and at different concentrations and time intervals, and their proliferation was assessed by XTT assay. Data are the average results of three independent experiments. *Asterisk* IC_{50} dose of exendin-4 in MCF-7 breast cancer cells was detected 5 μM/ml at 48th hour

time PCR analysis showed that *caspase-9*, *Akt*, and *MMP2* expression was reduced in dose group cells, compared with the control group cells. *p53*, *caspase-3*, *caspase-8*, *caspase-10*, *BID*, *DR4*, *DR5*, *FADD*, *TRADD*, *PARP*, *PTEN*, *PUMA*, *NOXA*, *APAF*, *TIMP1*, and *TIMP2* expression was increased in dose group cells, compared with the control group cells (Fig. 2a, b; $p<0.05$). Akt, *BCL-XL*, *BCl-2*, and *MMP-9* messenger RNA (mRNA) expressions were found statistically insignificant ($p>0.05$).

Migration and invasion assay

By Matrigel invasion chamber assay, the cell invasion was significantly inhibited in the dose-treated group, compared with the control group. Invasive cells were shown in Fig. 3a. Data of invasion % of the two groups were shown as follow: control group (58±8.04) % and dose group (37±5.2) % (Fig. 3b).

Colony formation assay

Colony formation analysis was performed by using colony formation assay. It is observed that the colony formation decreased in the 5 μM exendin-4-treated group, compared with the control group (Fig. 4a). Data of colony numbers of the two groups were shown as follows: control group (525.3±12.34) % and exendin-4 group (380.3±11.15) % (Fig. 4b).

Wound-healing assay

Effects of exendin-4 on cell migration were detected by wound-healing assay. According to results, exendin-4 reduced cell migration in MCF-7 cells, compared with the control group. Images at 0, 16, and 24 h were given in Fig. 5.

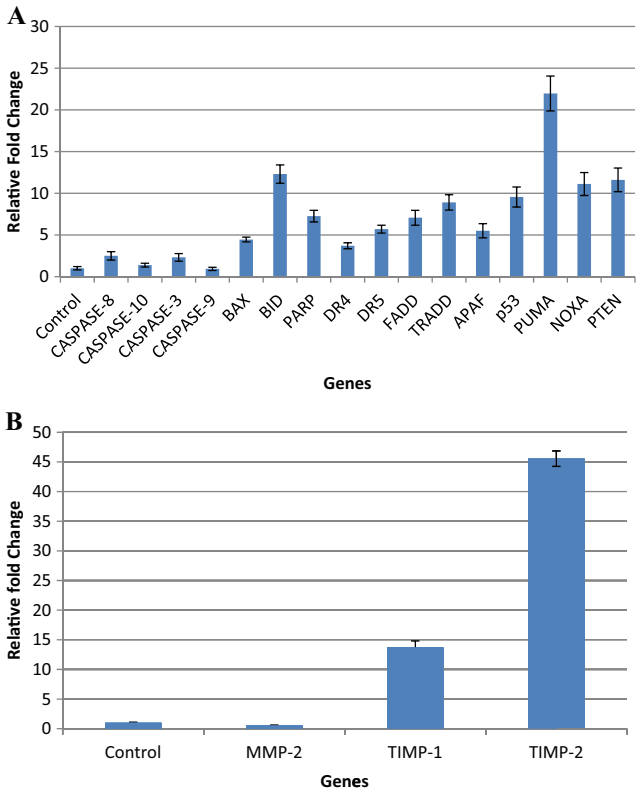

Fig. 2 **a** The mRNA expressions of genes relative to GAPDH mRNA expression were studied on real-time RT-PCR. The expression of these genes reduced in the exendin-4-treated cells, compared with the control cells. **b** Invasion related to mRNA gene expression change was demonstrated in the graph. These given mRNA fold changes were found statistically significant ($p<0.05$). mRNA changes of genes which were found statistically insignificant ($p>0.05$) were not shown

Discussion

We report in this article that exendin-4 significantly inhibits the proliferation of MCF-7 breast cancer cells and induces apoptosis via the modulation of apoptosis-related genes which play a role in extrinsic pathway and cell survival genes. Furthermore, effects of exendin-4 on cell migration, cell invasion, and colony formation capacity were evaluated in this study. It was shown that it inhibits migration, cell invasion, and colony formation capacity of breast cancer cells.

Recent advances about incretin-based therapy including GLP-1R agonists and dipeptidyl peptidase-4 inhibition have enabled popularity in diabetes treatment throughout the world [18]. Incretin therapy has many advantages such as pancreatic β cell protection, lower risk of weight increase, and less hypoglycemic attacks [19]. Incretin therapy is also expected to have effects in tissue-conservation beyond its glucose-lowering capability [20, 21]. Recent studies demonstrated that exendin-4 has pleiotropic effects that extend beyond its hypoglycaemic activity and anticarcinogenic potential effects [9, 16, 17, 21].

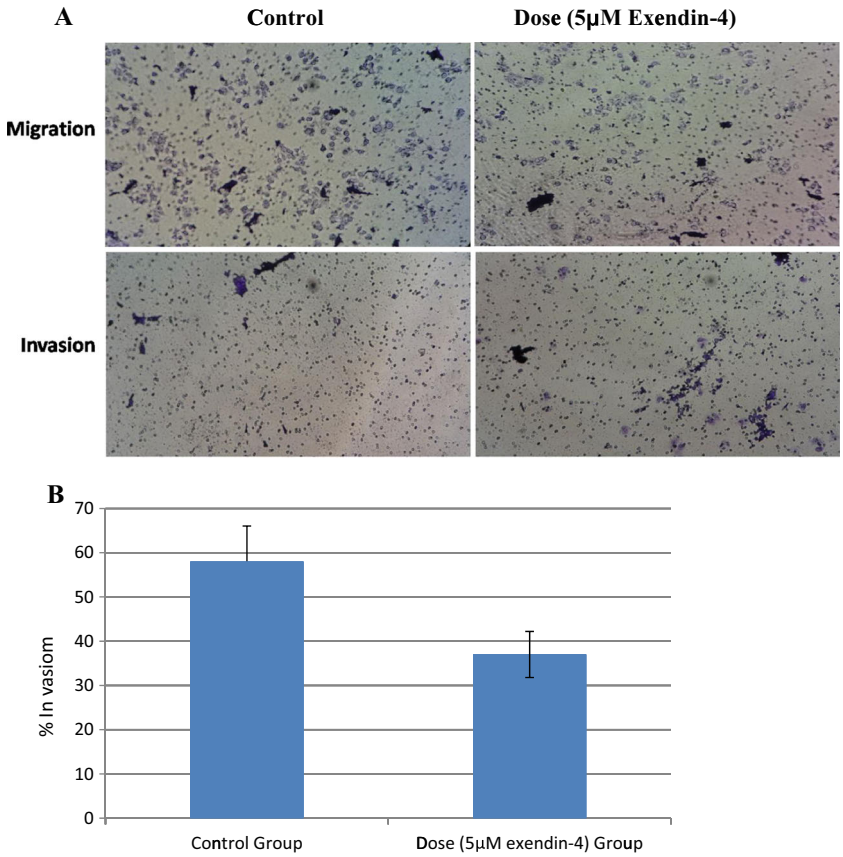

Fig. 3 **a** Migration and invasion assay of MCF-7 cells. Cells that passed through the membrane were counted in ten representative areas. **b** Summary graph for invasion were also shown respectively. Data was presented as mean±SD. $n=3$, $^*p<0.05$

In a previous study, it is showed that exendin-4 has anticarcinogenic effects in CT26 murine colon cancer cells by increasing increase intracellular cAMP levels and inhibiting glycogen synthase kinase 3 and ERK-MAPK activation, decreasing colony formation, and modulating apoptosis [17]. Luciani et al. defined the effects of exendin-4 on cell adhesion, differentiation, and migration of neuroblastoma cells. They demonstrated that GLP1-R stimulation inhibited migration of cells independently of the chemotactic stimulus used and also exendin-4 inhibited cell migration via IGF-1 and PDGF induction [16]. It has recently showed that exendin-4 has antitumor potential effects associated with production of cAMP in breast cancer [9]. Unlike previous *studies*, we focused on evaluating of exendin-4 and its molecular effect mechanisms related to apoptosis in MCF-7 cells. Invasion and migration are key process of tumor progression and closely related to breast cancer recurrence. Therefore, exendin-4 and its effects with underlying molecular mechanisms on invasion and migration were studied. Genes which play a role in extrinsic and intrinsic pathway of apoptosis were analyzed by RT-PCR and according to results, exendin-4 induced expressions of extrinsic pathway genes. Furthermore, exendin-4 reduced cell invasion by downregulating *MMP2* and upregulating *TIMP1* and *TIMP2* mRNA expressions.

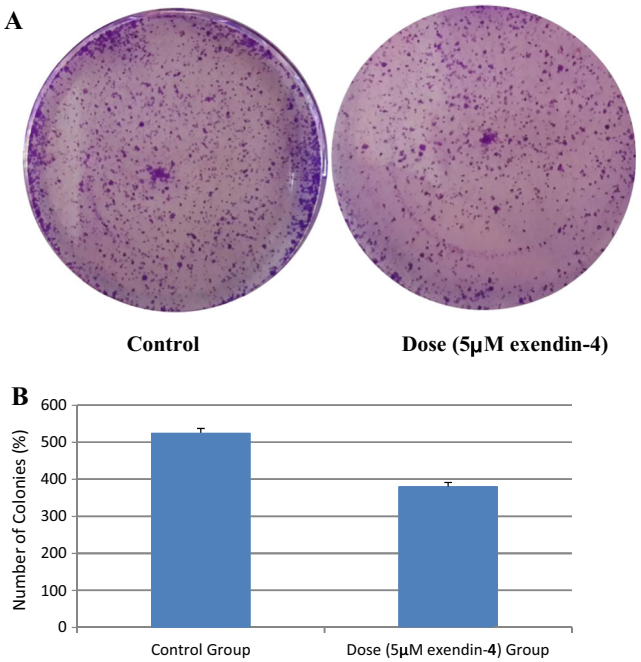

Fig. 4 **a** Exendin-4 decreases MCF-7 cell colony formation. Colonies were stained with crystal violet. **b** The number of colonies was significantly decreased in cells treated with exendin-4 compared with the control cells. Data are expressed as mean±SD, $n=3$, $^*p<0.05$

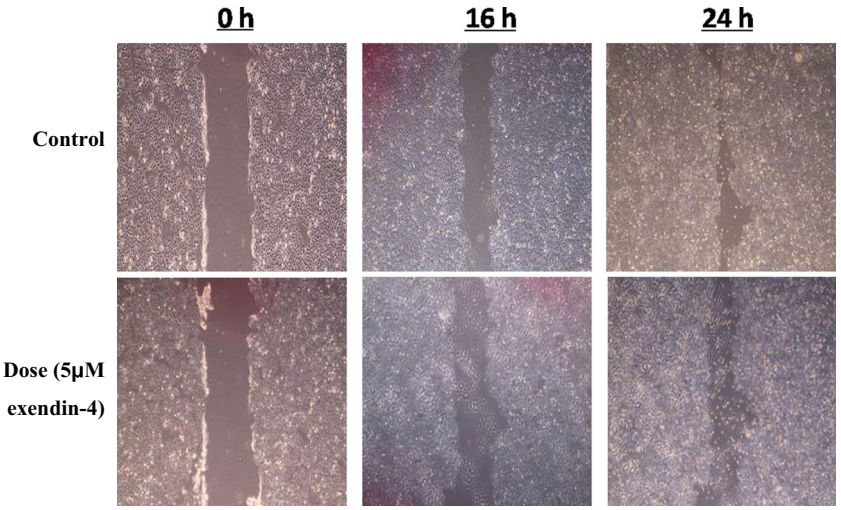

Fig. 5 Wound-healing assay results showed that exendin-4 reduced cell migration. Control and dose (5 μM exendin-4) images at 0, 16, and 24 h were given

In many studies done through breast cancer patients, the results showed that obesity and T2DM have been related to breast cancer risk and poor prognosis [4–9]. DM and cancer share many common mechanisms, containing increased insulin and insulin-like growth factor (IGF) signaling, dysregulation of ovarian steroid hormones, and chronic inflammation [22–24]. Besides, other factors may be associated with T2DM being correlated with decreased postprandial secretion and GLP-1 activity [25–28]. Decreased GLP-1 levels which exist in diabetes may play a role between diabetes and increased risk of breast cancer [9].

Because of T2DM incidence increase day by day around the world, exposure to GLP-1R agonists may affect a lot of women. Studies about the effects of GLP-1R agonists on malignant diseases have been limited. Ligumsky et al. suggested in their study that there are not any association between GLP-1 agonists and the risk of breast cancer; conversely, GLP-1 agonists lead to proliferation, migration, cell invasion, and colony formation inhibition [9]. Due to complex molecular signaling pathways such as cell growth, survival, and invasion in breast cancer, the effects of GLP-1 on these pathways should be studied more in detail.

In conclusion, molecules that have been studied as potential anticancer agent for the improvement of treatment options are one of the most important research issues. For the determination of these novel agents, the molecular mechanisms related to apoptosis and cell survival of breast cancer cells need more studies. According to our results about the effects of exendin-4, this agent significantly inhibits the proliferation of MCF-7 breast cancer cells and induces apoptosis via the modulation of apoptosis-related genes which play a role in extrinsic pathway and cell survival genes. Furthermore, effects of exendin-4 on cell migration, cell invasion, and colony formation capacity were evaluated in this study. As a result, exendin-4 may be a novel potential agent and may play a role in the investigation of therapeutic strategies for breast cancer. Therefore, more studies should be designed to find optimal safety dose of exendin-4 and to elucidate to underlying molecular effect mechanism.

Compliance with ethical standards

Conflicts of interest None

Ethics approval and consent to participate There is no need to take ethical approval and informed consent for this study.

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