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High Glucose-reduced Apoptosis in Human Breast Cancer Cells Is Mediated by Activation of NF-κB

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ABSTRACT

Tumor cells rely on glycolysis for their energy supply with the production of lactate even in normoxia condition, which is named aerobic glycolysis or Warburg effect. Therefore, high glucose (HG) concentration provides a favorable condition for increasing proliferation, angiogenesis and decreasing apoptosis, but its molecular mechanisms are still unknown. The objective of this study is to investigate HG condition on tumor cells behavior including proliferation, apoptosis, and an angiogenesis mediator.

In this study, MCF-7 derived from human breast adenocarcinoma, were cultured in DMEM with two different concentrations of glucose for 48 h (5.5 mM as normal glucose (NG) condition and 25 mM as HG condition). We used *Zingiber officinale* extraction for the inhibition of NF-κB. Cell proliferation assay was done by direct counting, cell viability by MTT method, bcl-2 by Immunocytochemistry, apoptosis by Hoechst/PI double staining and vascular endothelium growth factor (VEGF) by ELISA.

Results showed that HG increased lactate production, significantly. HG increased cell proliferation, cell viability, VEGF secretion, and bcl-2 expression while it decreased apoptosis. However, when HG was combined with *Zingiber officinale* extraction, cell proliferation, cell viability, VEGF secretion and bcl-2 expression decreased and apoptosis increased significantly due to inhibition of NF-κB.

Results revealed that HG increased cell proliferation, angiogenesis and decreased apoptosis due to activation of NF-κB pathway. Moreover, the probable mechanism of the activation of NF-κB in HG is increasing reactive oxygen species (ROS) in this condition that can activate NF-κB directly.

Keywords: Aerobic glycolysis; High glucose concentration; NF-Kb; Warburg effect

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INTRODUCION

Breast cancer is the most common cancer in women worldwide. In addition, nearly one percent of cancer in men is breast cancer. Despite many researches on different aspects of this malignancy, there are no

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effective methods of fighting with this disease.[1]

The main source of energy in tumor cells is glucose. The cells transport more extracellular glucose into the cytosol by up-regulation of glucose transporters (GLUT) expressions.[2] As mentioned, glucose is the main source of energy for cancerous cells' proliferation. Therefor hyperglycemia provides a desirable environment for the growth and survival of breast cancer cells by the induction of glycolysis.[3] Hyperglycemia has been reported to promote pancreatic and breast cancer cells in a dose-dependent manner.[1] It has been reported that cancer-specific survival of patients with hyperglycemia –like diabetic patients- was much less than that of patients with normoglycemic levels.[4] Furthermore, the results of previous studies showed that hyperglycemia confers resistance to chemotherapy in malignant breast cancer cells but not in non-malignant cells.[5]

Cancerous cells depend on glycolysis to generate energy with the production of lactate even with the presence of oxygen which is named aerobic glycolysis or Warburg effect.[3] This does not happen in normal cells to achieve their energy mainly through mitochondrial oxidative phosphorylation without the production of lactate.[6] However, aerobic glycolysis has lower efficiency in producing energy (ATP) from glucose in comparison to oxidative phosphorylation. Therefore, glucose is an essential factor in cancerous cells proliferation, regulating the expression of various proteins involved in angiogenesis, apoptosis, and autophagy.[3] There is evidence that higher rate of aerobic glycolysis is correlated with more aggressive manner in tumor cells. In other words, more aggressive tumors produce more lactate.[7,8]

In many cancers, the transcription factor of NF-κB is in abnormal modes of regulation and shows increased activity.[9,10] Recent researches have established a crucial role for NF-κB in cancer cell metabolism, tumor cell survival, regulation of cell cycle ,and proliferation, cellular adhesion, angiogenesis ,and development of drug resistance in cancer cells during therapy.[11,12] Some of important target genes of NF-κB are Cyclin D1 (involved in cell proliferation), [13-16] bcl-2 (cell apoptosis mediator),[16,17] and vascular endothelium growth factor (VEGF) (angiogenesis inducer)[16,18] that regulates many aggressive behaviors of tumor cells. There are natural herbs which have potential constituents for inhibition of NF-κB. For example, Zingiber officinale (Ginger) extraction is a well-known inhibitor for NF-κB.[19-21]

As noted, high concentration of glucose (HG) can promote tumor cell proliferation and more aggressive manner in comparison to normal glucose (NG) concentration. Our hypothesis was that HG could increase aerobic glycolysis rate and led to cancer promotion in this condition. Therefore, we decided to study the effect of HG on proliferation, apoptosis, and angiogenesis with the focus on the role of NF-κB in breast cancer MCF-7 cell line. For showing the role of NF-κB, was used from its natural inhibitor, *Zingiber officinale* (ZO).

MATERIALS AND METHODS

Chemicals

DMEM, fetal bovine serum (FBS), antibiotic solution (penicillin and streptomycin) and trypsin–EDTA solution were purchased from Gibco (Gibco BRL, Grand Island, NY, USA). Bovine serum albumin (BSA), Hoechst stain, propidium iodide (PI), Thiazolyl Blue Tetrazolium Blue (MTT), Dimethyl sulfoxide (DMSO), Triton-X100 and Glucose were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). *Zingiber officinale* ethanol extraction was purchased from Yas Daru Company (Tehran, Iran).

Cell Culture and Treatment

MCF-7 (purchased from Pasture Institute, Tehran, Iran), derived from human breast adenocarcinoma, were cultured in DMEM supplemented with 10% FBS and antibiotics (penicillin, 100 IU/mL and streptomycin, 100 µg/mL) in 5% CO_2 at 37°C. For treatment, cells were cultured in normal glucose concentration (5.5 mM+ 1mg/mL BSA for osmotic balance), high glucose (25 mM+ 1mg/mL BSA for osmotic balance) and high-glucose+*Zingiber officinale* extraction (HG+ZO) conditions. In order to obtain *Zingiber officinale* extraction IC_{50}, were used various concentrations, including 0, 25, 50, 100, 150 and 200 µg/mL. IC_{50} values were calculated as the concentrations that show 50% inhibition of proliferation on tested cell line. All investigations were done after 48 h.

Lactate Assay

For lactate assay, was used colorimetric lactate kit (Biorex Diagnostics, United Kingdom). Briefly, MCF-7 cells were seeded in 6-well plates. After 24 h, culture medium was exchanged and cells undergo to two treatments (NG and HG) for 48 h. Then the supernatant

of cell culture were collected and lactate assay were done according the kit manual.

MTT Cell Viability Assay

MTT assay is a colorimetric assay for assessing cell metabolic activity. NAD (P) H-dependent cellular oxidoreductase enzymes that largely in the cytosolic compartment may, under defined conditions, reflect the number of viable cells. Therefore, reduction of MTT and other tetrazolium dyes depends on the cellular metabolic activity due to NAD (P) H flux. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Briefly, cells were counted and seeded in 96-well cell culture plates with cell density of 5×10^3 cells/well. Cells were allowed to attach and were cultured in normal-as well as in high-glucose conditions for 48 hours. Then, cells were washed with PBS and incubated with MTT (5 mg/mL) for 4 h at 37°C. Cells were again washed with chilled PBS and DMSO (150 µL) was added in each well to dissolve the formazan crystals. Absorbance was measured at 595 nm using a spectrophotometer.

Direct cell Counting

MCF-7 breast cancer cells were seeded at 3×10^5 cells in 25 cm^3 cell culture flasks and were allowed to attach for 24 h. After 48 h of treatment (normal- as well as in high-glucose and high-glucose+*Zingiber officinale* extraction conditions), Cells were trypsinized and resuspended in equal volumes of medium and Trypan blue (0.05% solution) and counted using a haemocytometer.

Immunocytochemistry (ICC)

MCF-7 cells were seeded in 96-well plates. After 24 h, culture medium was exchanged and cells undergo to three treatments (NG, HG and HG+ZO) for 48h. The cells were washed with PBS and then fixed with 4 % paraformaldehyde for 10 min at room temperature followed by blocking (Bovine Serum Albumin 1%) and permeabilized by using Triton X-100. Endogenous peroxidase activity was blocked by 3% H_2O_2 in methanol for 30 minute. The cells were then incubated overnight at 4°C with mouse anti-bcl-2 monoclonal antibody (Santa Cruz, United States) as primary antibodies. Thereafter, the cells were washed with PBS three times and incubated for 2 h with anti-mouse antibody conjugated with horseradish peroxidase (HRP). For qualitative detection of bcl-2, the cells were stained with 3, 3′-Diaminobenzidine (DAB) that positive cells were brown. For quantitative detection of bcl-2, we used luminol that when exposed to HRP and H_2O_2, it will be emitted and this can detected by luminometer (Promega, United States). Staining cells without primary antibody utilized as negative control (Data not shown).

Apoptosis Assay

Apoptosis was studied by Hoechst/PI double staining method. This method is a rapid and convenient assay for apoptosis based upon fluorescent detection for the compacted state of the chromatin in apoptotic cells. Hoechst 33342, a kind of blue-fluorescence dye, stains the condensed chromatin in apoptotic cells more brightly than the chromatin in normal cells. Propidium iodide (PI) is a red-fluorescence dye which is only permeant to dead cells. Briefly, cells were seeded and treated in a 24 wells cell culture plate for 48 h. After treatment, the cells were washed by PBS and then incubated by Hoechst 33342 (10 µg/mL) for 7 minute at 37 °C. After incubation by Hoechst 33342, cells were incubated by PI (2.5 µg/mL). The plates were maintained in the dark for 15 minutes. After incubation, cells were washed by PBS and were observed immediately under invert fluorescence microscope (Nikon, Japan).

VEGF ELISA

For quantitative assay of VEGF, we used Human VEGF DuoSet ELISA kit (R&D system). Briefly, cells were seeded in a 24 well plate in DMEM with 10% FBS for 24 h. After 24 h, the media was exchanged with treatment media, as previously mentioned. After 48 h of treatment, sample were collected. VEGF were measured according to the manufacturer's instructions and then using a micro-plate reader, read the plate at 450 nm wavelength. All data were normalized to cell count obtained before cell lysis. Results were expressed as pg VEGF/mL/5×10^4 cells. Each sample was measured in triplicate.

Statistical Analysis

All values are expressed as mean±standard deviation. Results shown are representative of three different experiments. Statistical analyses were performed using the ANOVA and Student's t-test. A $p<0.05$ was considered to indicate a statistically significant difference. Statistical calculations were

performed using SPSS software 16.0 (SPSS, Chicago, IL, USA).

RESULTS

Effect of High Concentration of Glucose on Aerobic Glycolysis

Lactate is the end product of aerobic glycolysis. Direct quantification of lactate can show the rate of aerobic glycolysis in tumor cells. Our results demonstrate lactate production in HG condition was significantly higher than NG condition. These data revealed that HG can promote Warburg phenomenon (aerobic glycolysis) (Figure 1A).

Effect of High Concentration of Glucose on Cell Proliferation and Cell Viability

Cell proliferation assay showed that in MCF-7 cells, HG condition promoted cell proliferation compared with a normal glucose condition. The IC_{50} of *Zingiber officinale* extraction according to MTT method was obtained 50.2 µg/mL. When HG combined with *Zingiber officinale* extract (HG+ZO), cell proliferation was reduced, significantly. However, there was no significant difference between NG and HG+ZO condition in cell proliferation (Figure 1B). In addition, the MTT assay demonstrated that in MCF-7 cells, a HG condition led to increased cell viability. Like cell proliferation, when HG combined with *Zingiber officinale* extract, cell viability was decreased in compare to HG condition. Also, there is no significant difference between NG and HG+ZO condition in cell in cell viability (Figure 1C). These data revealed that *Zingiber officinale* extract can inhibits the cell proliferation and viability according to inhibition of NF-κB.

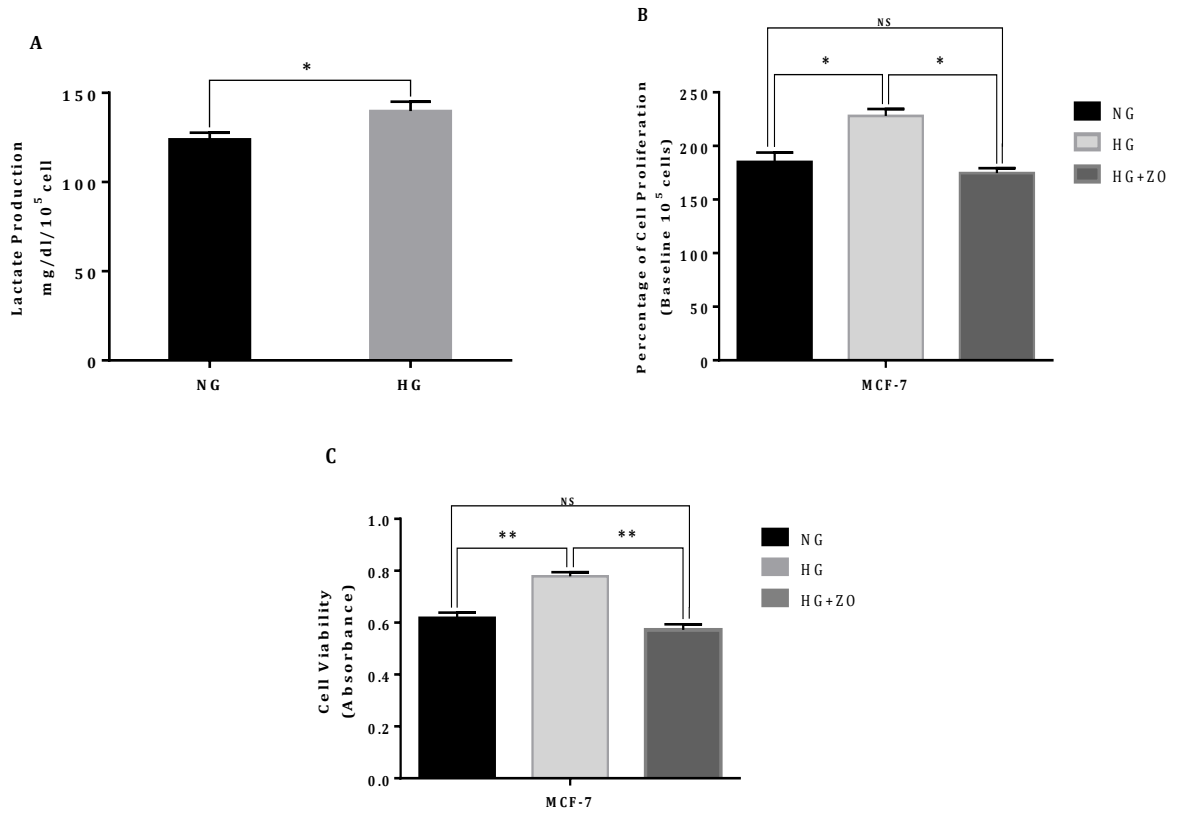

Figure 1. A) Aerobic glycolysis rate by lactate as final production in different conditions of medium after 48 h. B) Percentage of cell proliferation in different medium condition after 48 h. According to this data, high glucose (HG) condition can promote proliferation in compare to normal glucose (NG) condition. When HG combine with *Zingiber officinale* (ZO) extraction, proliferation was decreased significantly. This reduction clarify the role of NF-κB in cell proliferation. C) Cell viability (MTT) in different condition after 48 h. It can be observed HG can elevated cell viability but when it combined with ZO extraction, viability was reduced significantly. NS: not significant; *p<0.05; **p<0.01

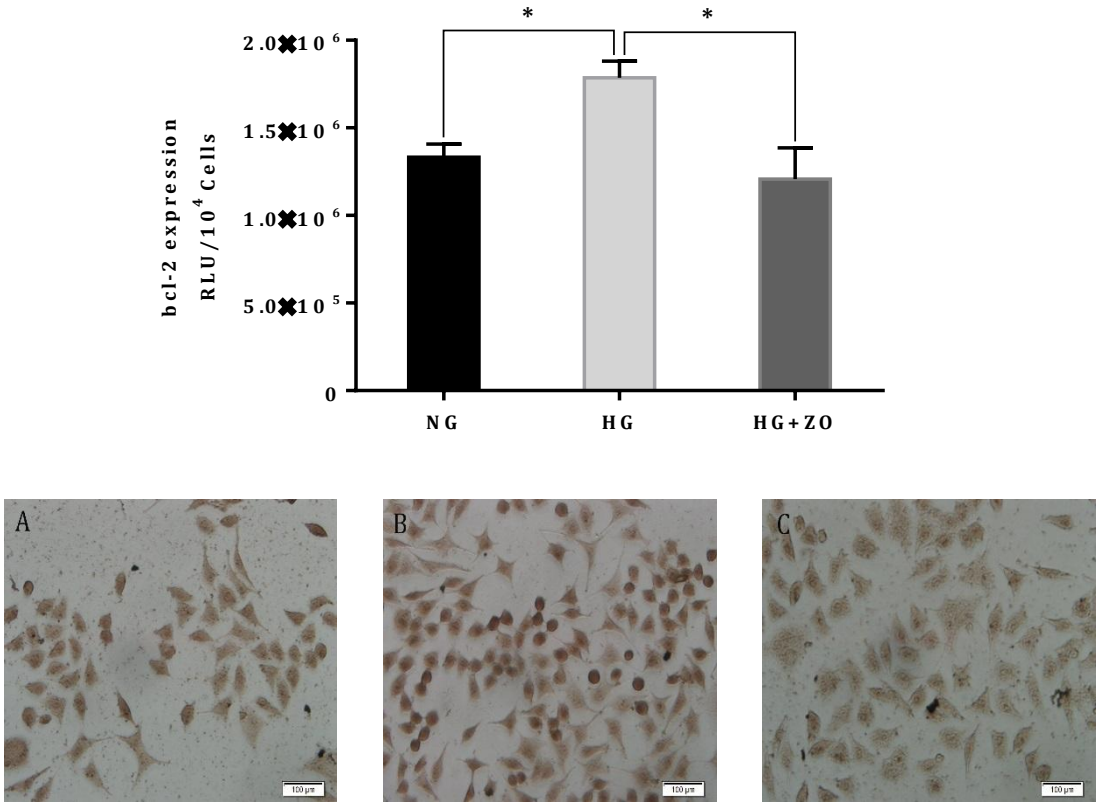

Figure 2. The chart presents the quantitative bcl-2 expression in MCF-7 breast cancer cell line by luminometric assay after 48 h. Images shows the qualitative assay of bcl-2 expression. A) bcl-2 expression in MCF-7 cell line in normal glucose (NG) condition. B) bcl-2 expression in MCF-7 cell line in high glucose (HG) condition. C) bcl-2 expression in MCF-7 cell line in HG+ *Zingiber officinale* (ZO) condition. *p<0.05.

Effect of High Concentration of Glucose on Expression of bcl-2

bcl-2 protein is one of the most important regulator of apoptosis according to its role as an anti-apoptotic protein. NF-κB is an important regulator for expression of this protein.[16,17] Our results showed HG condition increased expression of anti-apoptotic bcl-2 protein in compare to NG condition after 48 h. When HG combined with *Zingiber officinale* extract, the expression of bcl-2 protein was decreased (Figure 2). These data revealed increasing expression of bcl-2 in HG condition probably regulates by NF-κB/bcl-2 pathway.

Effect of High Concentration of Glucose on Apoptosis

One of the assay methods for investigation of apoptosis is usage of double staining with Hoechst 33342/PI. In this method normal cell is blue while the apoptotic cell is brightly blue and may be degraded. Dead cells will be red by PI staining due to lost nuclear membrane integrity. Our results revealed HG condition decreased apoptosis in compare to NG condition significantly (p< 0.01). Also, combination of HG and *Zingiber officinale* extract could increase apoptosis, significantly (Figure 3). These data is consistent with bcl-2 expression in the same conditions.

Effect of High Concentration of Glucose on VEGF

VEGF is an important mediator of angiogenesis which secreted from tumor cells. Our results showed HG condition caused the increase of VEGF secretion in MCF-7 cell line. However, when HG combined with *Zingiber officinale* extract, VEGF was significantly decreased (Figure 4). These data showed HG could increase angiogenesis inducer.

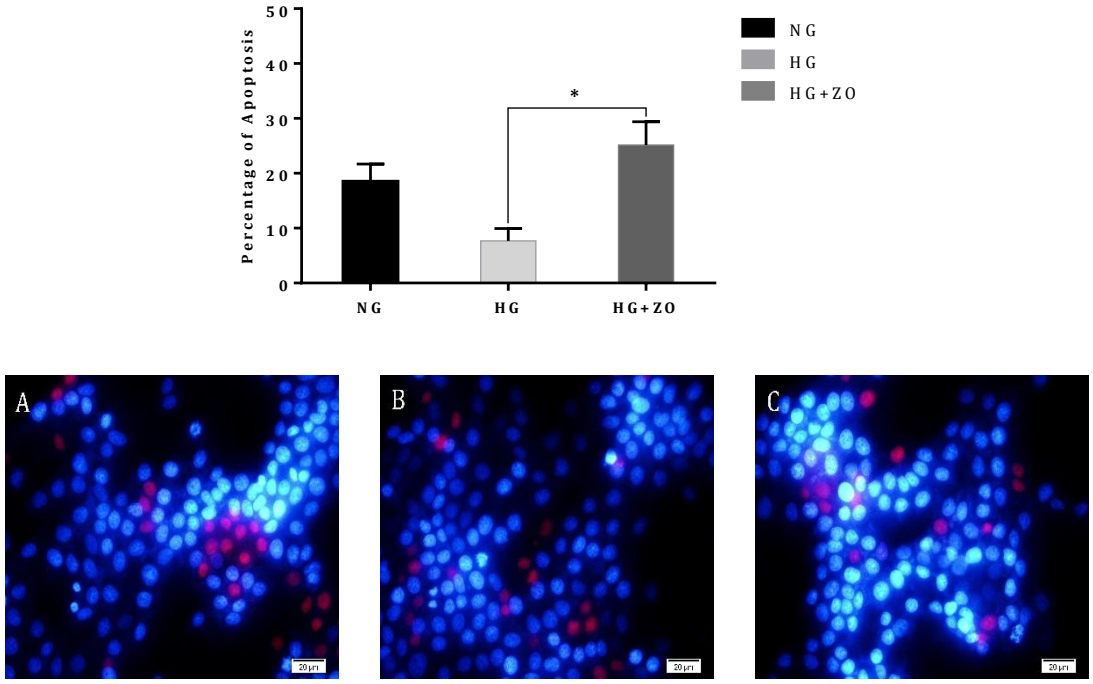

Figure 3. The chart presents the percentage of cell apoptosis in different condition of medium after 48 h by counting. Images show the apoptosis (brightly blue or degraded) and dead cells (red). A) Cells in normal glucose (NG) condition. B) Cells in high glucose (HG) condition and C) Cells in HG+ *Zingiber officinale* (ZO) condition. *p<0.05.

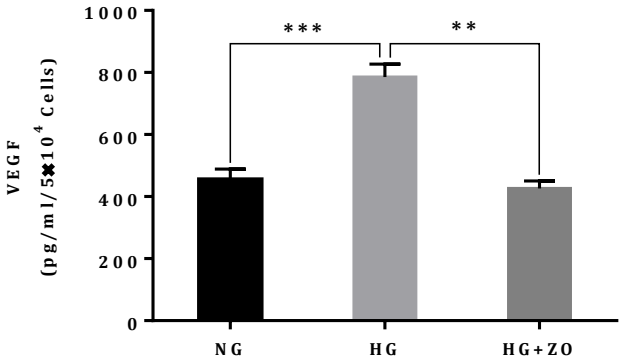

Figure 4. The chart presents the angiogenesis factor -*e.g.* vascular endothelium growth factor (VEGF)- secretion of MCF-7 cell lines in different conditions after 48 h. NG: normal glucose; HG: high glucose; HG+ZO: High glucose+ *Zingiber officinale* extraction; **p<0.01, ***p<0.001.

DISCUSSION

It is well-recognized that hyperglycemia can promote the development of several cancers, including breast cancer. However, the exact involved mechanisms still remain to be elucidated.[22] Here, is shown that HG can significantly promote cell proliferation and angiogenesis and decrease apoptosis of MCF-7 breast cancer derived cells.

Our results showed that HG can promote Warburg phenomenon (aerobic glycolysis) with higher production of lactate. as well as, was shown that

aerobic glycolysis correlated with promotion of cell proliferation, survival, angiogenesis ,and metastasis.[23,24] In other words, more aggressive tumors show higher aerobic glycolysis rate and more lactate production and secretion in comparison to less aggressive tumors or normal tissues in the same conditions.[23,24] For example, aerobic glycolysis and lactate production of MDA-MB-231 cells (an invasive and late stage breast cancer cell line) are more than MCF-7 cells (non-invasive and early stage breast cancer cell line) in the same conditions.[7,8] It is noteworthy, that secreted lactate through cancer cells enhances angiogenesis due to the activation of NF-κB/IL-8 pathway in adjoining endothelial cells. In summary, endothelial cells are uptake lactate by Monocarboxylate Transporter 1 (MCT1) and these lactate can activate NF-κB by activation of IKK/IκBα/NF-κB classical pathway (Figure 5).[25]

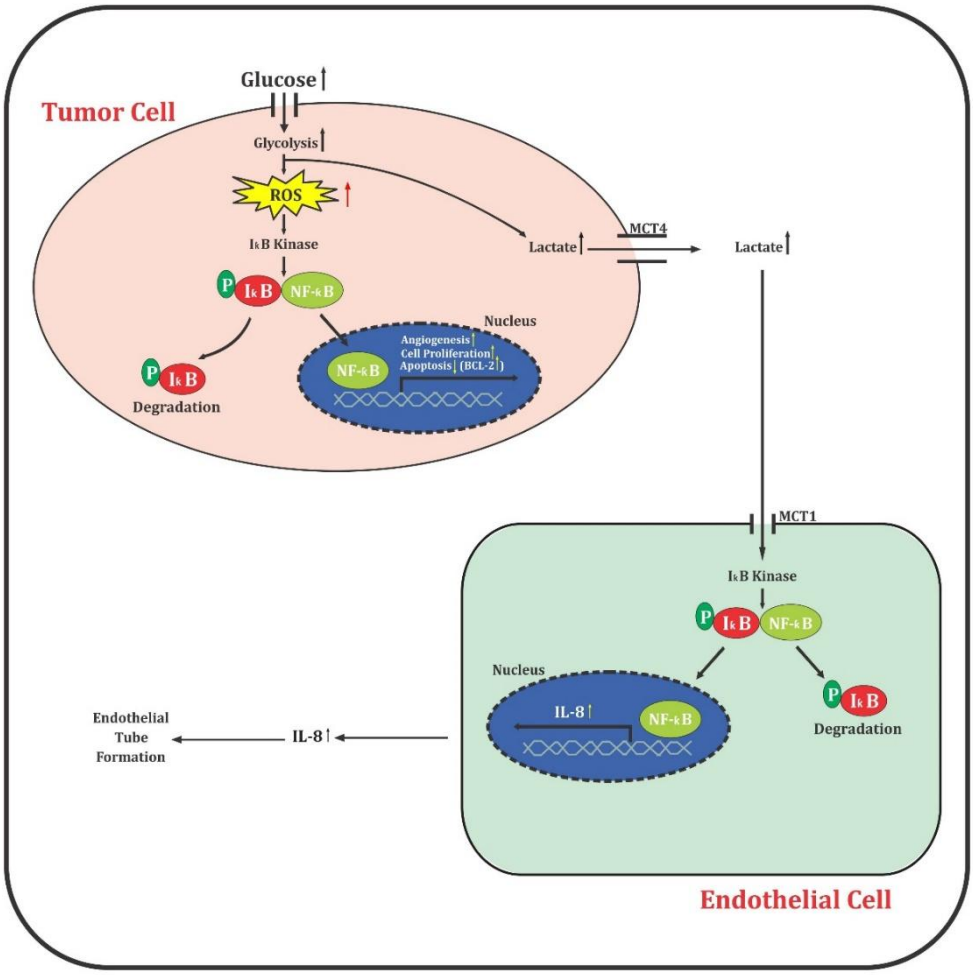

Figure 5. The scheme presents the role of high glucose (HG) concentration in increasing production of reactive oxygen species (ROS) and lactate. ROS in the tumor cells activates the NF-κB by activation of IKK/IκBα/NF-κB classical pathway. Activated NF-κB translocate to the nucleus and up-regulate its target genes such as genes involved in cell proliferation (like Cyclin D1), angiogenesis (like vascular endothelium growth factor (VEGF)) and anti-apoptosis (like bcl-2). Also, in tumor cells, increased lactate is exported to tumor microenvironment by Monocarboxylate Transporter 4 (MCT4). Adjoining endothelial cells are uptake these lactate by Monocarboxylate Transporter 1 (MCT1). It can be an activator for NF-κB. Therefore, activated NF-κB in endothelial cells translocate to the nucleus and up-regulate the IL-8 which involved in endothelial tube formation, an important event in angiogenesis.

It was shown that Warburg phenomenon is correlated with more steady-state Reactive Oxygen Species (ROS) in cancer cells.[23] There are many studies that show HG can promote the generation of ROS in different cell lines as well as MCF-7 breast cancer cells.[1,26-28] Our results can explain the possible mechanism of induced ROS by high glucose concentration. In this study, it is proposed that HG accelerates aerobic glycolysis and this acceleration is the cause of increased generation of ROS in cells.

One possible mechanism for increasing proliferation and angiogenesis and the inhibition of apoptosis (by increasing of bcl-2 protein) in the HG is the activation of NF-κB pathway by ROS.[18] It has been shown that ROS can activate NF-κB by the activation of IKK/IκB*α*/NF-κB classical pathway.[29] Activation of NF-κB translocates it into nucleus, where it acts as a transcription factor to induce the expression of proliferative, angiogenesis and anti-apoptotic genes (Figure 5).[13-18] Although the most important aspect of NF-κB activation is nuclear translocation, additional post-translational modification of NF-κB itself and its surrounding chromatin environment is also critical for enlisting the transcriptional apparatus and stimulating target gene expression. Previous studies showed that phosphorylation and acetylation of NF-κB are important post-translational modification for NF-κB activation.[30-33] Therefore, it does not seem that the evaluation of the amount of nuclear NF-κB or phosphorylated-NF-κB shows the activity status of this transcription factor. So, one way for assessing the activity of this transcription factor is the quantification of downstream target genes.[32] In this study, to demonstrate the role of NF-κB in increasing proliferation and angiogenesis, as well as decreasing apoptosis in HG, the extract of *Zingiber officinale* was used as a well-known natural NF-κB inhibitor. This extract inhibits NF-κB activity by the inhibition of IKK/IκB*α*/NF-κB.[19-21] The results of the present study revealed when *Zingiber officinale* extract was combined with HG, it significantly decreased the expression of bcl-2 and VEGF. Also, proliferation decreased and apoptosis increased in comparison to high glucose concentration. However, these parameters haven't significant difference in *Zingiber officinale* extract was combined with HG in comparison to NG condition. These data showed the role of activated NF-κB in HG.

In this study, it was demonstrated that HG can significantly increase cell proliferation and viability by direct counting and MTT methods. However, when *Zingiber officinale* extraction was combined with HG, both proliferation and viability decreased significantly. The data revealed the probable role of involved genes in proliferation which were regulated by NF-κB, like Cyclin D1. In the past, mitogenic, pro-migratory and pro-invasive effects of HG have been shown in both normal and cancer cells.[22] In confirming to our results, HG has been found to increase the proliferation of MCF-7 cells.[2,34-36] In previous studies, the effect of HG in cell cycle progression and cell proliferation in MCF-7 breast cancer cells was shown by increasing DNA synthesis significantly, Protein Kinase C (PKC) and Cyclin-dependent kinases 2 (cdk2)/Cyclin D1 expression.[35,36] Cdk2 and Cyclin D1 may have also important roles in neoplastic transformation and cell cycle progression through G1–S border.[35] In contrast, Gupta et al showed that HG inhibited proliferation and decreased Cyclin D1 in comparison to NG condition in MCF-7.[1]

Our findings showed that HG inhibited apoptosis in MCF-7 breast cancer cells, significantly. In accordance with this results, Zhu et al[36] showed that increasing concentration of glucose can inhibit apoptosis in a dose dependent manner in MCF-7 breast cancer cells. Also, Alaswad et al[2] achieved similar results. In the present study, one of the most important molecular mechanisms involved in apoptosis -*i.e.* the expression of bcl-2- was investigated. It was shown that high concentration of glucose causes increasing anti-apoptotic bcl-2 protein. However, when *Zingiber officinale* extraction was combined with HG, the expression of bcl-2 decreased significantly. These results can be explained as follow that bcl-2 is a gene regulated by NF-κB[16,17] and HG can increase expression of this gene by the activation of ROS/NF-κB/bcl-2 pathway.

Our results revealed that HG increased VEGF significantly in comparison to NG condition. However, when *Zingiber officinale* extraction was combined with HG, the expression of VEGF decreased significantly. It has been shown that VEGF expression is regulated by NF-Kb.[16,18] Thereby, it is possible that HG unregulated VEGF by the activation of ROS/NF-κB/VEGF pathway.

Our results revealed that HG, like hyperglycemia in diabetic patients, increases proliferation and angiogenesis and inhibits apoptosis in MCF-7 breast

cancer cells. The inhibition of apoptosis in this condition can be due to the increase in bcl-2 protein. According to our results and results of other studies, it is possible that HG can make increasing of proliferation and angiogenesis and the inhibition of apoptosis by activating of ROS/NF-κB pathway.

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