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RESEARCH PAPER

The anti-diabetic effect of naonoliposmal encapsulated hybrid extract on RIN-5F diabetic cell line

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ABSTRACT

Objective(s): This study investigates the effect of naonoliposmal encapsulated hybrid extract (NEHE) obtained from three plants (green tea, garlic, and chicory) in treating diabetes through expression of PPARgamma, BGK, and GLUT2 genes involved in diabetes and their relationship with miR-27a.

Materials and Methods: Herbal extracts were encapsulated in liposome. RIN-5F cells were exposed to streptozotocin (STZ) for 24 hr and divided into five groups to induce the diabetes model. Three groups received 0.4, 1, and 2 mg/ml of NEHE, a positive control group received 5 µg/ml of metformin for 72 hr, and a negative control group was only treated with FBS. Then, they were subjected to an MTT assay to check the toxicity of the extract. An immunohistochemical test was performed to check the level of insulin expression in different groups. Real Time-PCR test was performed to check the expression of desired genes.

Results: MTT assay showed that the NEHE extract had no toxicity on the tested cells. Also, the immunohistochemistry results showed that insulin expression NEHE group was significantly higher than that of the metformin group ($P<0.001$), indicating the antidiabetic effect of the extract. In all groups treated with the extract, especially with the maximum extract concentration, the expression level of BGK, PPARgamma, miR-27a, and GLUT2 genes was significantly higher than that of the untreated control group. Furthermore, there was no significant difference in the expression of BGK and PPARgamma genes in groups trated with either metformin or plant extract at concentration of 2 mg/ml groups. NEHE showed an improved effect in treating diabetes in a dose-dependent manner by increasing insulin secretion from pancreatic cells.

Conclusion: As a glucose transporter and sensor, GLUT2 controls the balance between intracellular and extracellular glucose concentrations. When glucose-induced insulin secretion is impaired, garlic can increase the half-life of insulin, while green tea and chicory reduce insulin resistance through the miR-27a pathway.

Keywords: *BGK, Diabetes, GLUT2, Herbal extract, miR-27a, PPARgamma*

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INTRODUCTION

The prevalence and incidence of diabetes are increasing in many societies, especially in developing countries [1]. Many people have diabetes and related diseases such as obesity and cardiovascular and kidney diseases worldwide [2]. Although the standard drugs known for treating diabetes such as metformin are beneficial, their use is associated with side effects, and most of them cause digestive problems. Therefore, alternative treatments, especially herbal medicines known to lower blood sugar, have recently received attention. This study applied liposomal nanocarriers as a new method with many advantages for treating diabetic cells [3].

Diabetes is a heterogeneous disease in which genetic and environmental factors are both effective in causing this disease [4]. Three genes, glucokinase gene (BGK), glucose transporter GLUT2, peroxisome proliferator-activated receptor gamma (PPARγ), and a microRNA called miR-27a, play a role in causing this disease. BGK gene regulates blood glucose levels and its failure causes diabetes mellitus [5, 6]. GLUT2 is the major glucose transporter in hepatocyte and β-cells of pancreatic islets. It balances the glucose concentration inside and outside the cell and also appropriately controls glucose-sensitive genes expression in the liver [7]. When the amount of free fatty acids in the blood circulation increases and fat accumulates in

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non-fat cells, insulin resistance increases. Due to its ligands, PPARgamma receptor improves this condition by strengthening the storage of fatty acids in fat deposits, the expression and regulation of fat hormone secretion. Thus, this receptor plays a role in glucose homeostasis and improving glucose sensitivity in pancreatic ß-cells [8]. MiR-27a increases insulin sensitivity in its interaction with PPARγ, and this microRNA's high expression reduces glucose absorption and consumption [9].

Oxidative stress is one of the first defects that cause the development of diabetes. Some micro RNAs can regulate Nrf2 which is the master regulator of the cellular antioxidant [10]. Hence, the aim of this study is using herbal antioxidants to treat diabetes, which can be regulated through Mir- 27a.

This study uses the nanoliposome combination of green tea, chicory, and herbal garlic with strong antioxidant properties to treat diabetes, which is regulated through miR-27a. This nanoliposomal formulation was used for the treatment through impacting on genes involved in diabetes incidence due to their known role in lowering blood sugar. Nanoliposomes benefit advantages such as *in vivo* and *in vitro* stability, as well as lack of unwanted interactions with other molecules. Moreover, the performance of bioactive agent can be improved due to the enhanced bioavailability and solubility of nanoliposomes [11]. The green tea plant contains flavonoids that have similar effect to insulin and have insulin-enhancing activities [12]. Chicory has antidiabetic effects and can reduce the impact of associated diseases with type 2 diabetes [13]. Garlic also increases the secretion and sensitivity to insulin due to the presence of disulfides [14]. Therefore, this study aimed to evaluate the anti-diabetic effect of NEHE on rat pancreatic cells induced by STZ.

MATERIALS AND METHODS

Preparation of nanoliposome containing plant extract

Hydroalcoholic extract of the dried powder of each plant (100 g) was prepared [15]. The thin-layer film hydration method was used to prepare nanoliposome formulations. For this purpose, 5 ml of lecithin, cholesterol, and PEG 2000 (1, 2-Distearoyl-phosphatidyl ethanol amine- methyl- polyethylene glycol conjugate) (Merck, Germany) was dissolved in 5 mL of ethanol. Then, the thin layer was formed by evaporating the solvent in a rotary evaporator (Heidolph, Germany) at 50° under a vacuum [16]. The extracts of green tea, chicory, and garlic were dissolved in sterile distilled water (10 ml) and shacked for 30 min at 30 °C, until their final concentrations. Then the resulting solution was added to the obtained thin lipid layer. To increase the hydration efficiency of the thin lipid layer, the resulting mixture was placed on a stirrer device with glass beads with a diameter of 0.5 ml until the lipid layer disappeared. The obtained samples were sonicated for 15 min in an ultrasonic homogenizer UP200H (Hielscher, Germany). Then the homogenized suspension was taken for 1 hr in the vicinity of nitrogen gas at lipid phase transition temperature to homogenize the resulting suspension and produce nano-sized liposomes. Finally, the resulting suspension was centrifuged (Sigma, Germany) to obtain a clear suspension containing nanoliposomes carrying plant extract. The produced nanoliposome quickly sealed and stored in darkness at 4°C. Then, the morphological characteristics of NEHE were determined by SEM (TESCAN, Check). NEHE suspension was diluted 100-fold with double distilled water before measurements. The size and polydispersity (PI) of liposome nanoparticles were investigated by dynamic light scattering using a Zeta potential (surface charge potential), DLS (Horiba, SZ100, Japan) device, and the nanoparticle charge was measured by a Nano Zetasizer (Malvern, England).

Cell line culture and diabetes induction

RIN-5F cells (beta cells isolated from rat pancreatic tumor) were purchased from the Iranian Biological Resource Center and cultured in RPMI1640 medium containing L-glutamine (2 mmol) and 10% fetal bovine serum at 37°C. Cells were cultured to 70% confluency. The medium of the cells changed every three days. Cells were trypsinized and centrifuged and were counted using trypan blue on a neobar slide. Fifty thousand cells were cultured in each well of a 24-cell plate (5 groups with three replicates), and the plates were placed in an incubator overnight. Cells were treated with streptozotocin STZ (CAS: 18883-66-4) for 24 hr with a final concentration of 30 mM to induce diabetes [17, 18]. After 24 hr, the diabetic cells, lacking the ability to secrete insulin, were subjected to different treatments.

In experimental groups 1 to 3, the cells were subjected to 0.4, 1, and 2 mg/ml of

NEHE, respectively. These concentrations were calculated based on the entrapment efficacy (EE) that plays a crucial role in assessing the effectiveness of entrapment methods, indicating the capacity of liposomes to hold substances. In order to determine the efficiency of the entrapment process, extract-loaded liposomes were isolated from the unentrapped extracts as explained in detail by Jahanfar *et al.* [19]. In the positive control group, diabetic cells were treated with metformin (5 µg/ml) [20-22]. In the control group, diabetic cells were only placed in a culture medium containing FBS. All cells were cultured in RPMI1640 medium with 5% glucose for 72 hr.

MTT assay

MTT assay was performed to investigate the cytotoxicity of NEHE. About 1×105 cells were cultured in 96-well plates, and after 24 hr, they were treated with the desired concentrations of the extract for 72 hr. Then, the supernatant was removed, and 100 µl of 0.1% MTT solution (Cat NO: DMA100, Sigma-Aldrich) was added to each well. After incubating for 3-4 hr at room temperature, the supernatant was removed, and 100 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich) (CATNO: BP231-100) was added to each well to dissolve the formazan crystals. After 15 min of incubation, the optical density (OD) was measured at 570 nm by a spectrophotometer (Thermo Fisher).

Immunohistochemical test

This test was used to determine the level of insulin expression after the induction of diabetes. Cells (5×10^6) were treated with metformin, and then fixed with 4% paraformaldehyde. The samples were washed 3× in PBS. The cells were permeabilized with 0.3% Triton (Sigma-T8787) for 30 min. The blocking agent was 10% Goat serum for 30 min at room temperature. The primary antibody (mouse monoclonal anticarboxymethyl lysine antibody, Orb248410, USA) was diluted (1/100) in BSA overnight and incubated in a fridge in a humidified chamber. Samples were washed 4 times in PBS and incubated (AriaTeb) with secondary (H+L) antibody (FITC، Orb688924) at dilution of 1/150 in BSA at 37 °C for 1 hr and 30 min at a dark place. Further, the samples were washed 3 times in PBS and exposed to DAPI (1/2000, D9542-Sigma) for 20 min at room temperature, then washed with PBS. Cells were examined under a fluorescence microscope (Olympus) using a ×400 objective. On each study slide, 200 cells were evaluated for confirmation of markers (antibody expression and nucleus staining).

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA of RIN-5F cells treated with different concentrations of NEHE for 72 hr was extracted by the Trizol reagent (Sigma-Aldrich) method. The extracted samples were placed at -20°C for short-term storage or -80°C for long-term storage. The quality and quantity of RNA were checked by UV spectrophotometry and a Nanodrop device. cDNA synthesis was done using the cDNA reverse transcription kit in the Dry block heater thermocycler device of Pars Toos company. The qRT-PCR reaction was performed using SYBR Green PCR Master Mix (Amplicon) and primers in Table 1. The final volume of each PCR reaction was 10 µl. *U6* and *GAPDH* genes were used as reference genes.

Statistical analysis

Data were presented as mean ± standard

Table 1. Primers used in the treatment of RIN-5F cells treated with different concentrations of NEHE

Primers	
r-BGK-f	GCGAGCCATGAGTGACTGAC
r-BGK-r	GACTCTGGGGCTCTAACCTCT
r-Glut2-f	CAATGCCACAGACACCCCAC
r-Glut2-r	ATCCCCAAGCCACCCACCAA
r-pparg F	CTCAGGCAGATTGTCACA
r-pparg R	CAGCGACTGGGACTTTTC
r-GAPDH-F	AGGTCGGTGTGAACGGATTTG
r-GAPDH-R	TGTAGACCATGTAGTTGAGGTCA
miR-27a-Stem Loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGGAA
miR-27a-forward	GGGGGGTTCACAGTGGCTAAGT
Univeral reverse primer	CCAGTGCAGGGTCCGAGGTA
Stem-loop U6	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATA
Forward U6	CCCGCAAGGATGACACGCA

deviation (SD) in 3 replicates. Data analysis was done by SPSS software version 23. One-way ANOVA test and then Tukey's post hoc test was used to analyze the data. The significance level was $P \leq 0.05$.

RESULTS

Characteristics of nanoliposomal extract

The mean particle size was 247.8 nm, and had a polydispersity index of 0.974. Zeta potential value was found to be -27 mv. Fig. 1a shows the morphology of nanoliposomal particles obtaind using SEM electron microscopy and Fig. 1b shows the mean size of particles by DLS (Dynamic Light scattering).

Cell culture

RIN-5F cells were cultured as control, metformin (5 mg/ml) and NEHE (0.4, 1, and 2 mg/ml) groups. Fig. 2 shows these cells before STZ, after STZ, and after NEHE treatments. After 72 hr of treatment with the NEHE, the morphology of the cells was spindle-shaped, and they were in good condition in terms of morphology and cell growth.

Examining the cytotoxic effect of the extract by the MTT method

The results of the MTT assay showed that after inducing diabetes followed by treating the cells with metformin (5 mg/ml), the cell viability decreased significantly compared to that of the control group ($P<0.05$). However, after 72 hr of treatment of cells with different doses of NEHE (0.4, 1, and 2 mg/ml), the percentage of cell survival was not significantly different from that of the control group. Moreover, by increasing the extract concentration, the cells

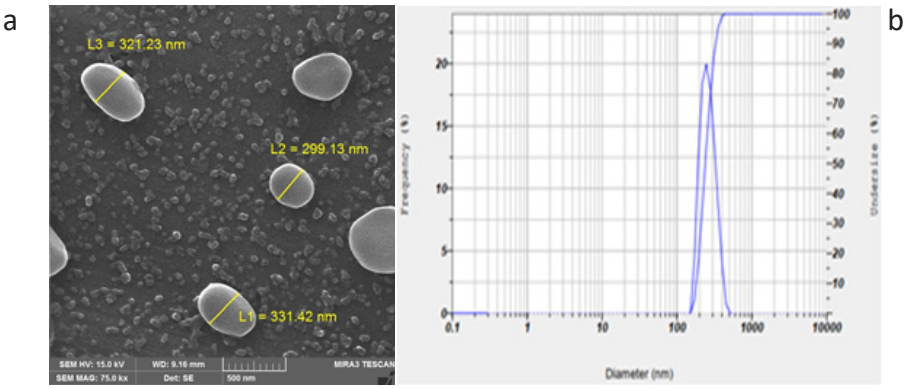

Fig. 1. Scanning electron micrograph of NEHE (a), the mean size of NEHE by DLS (b)

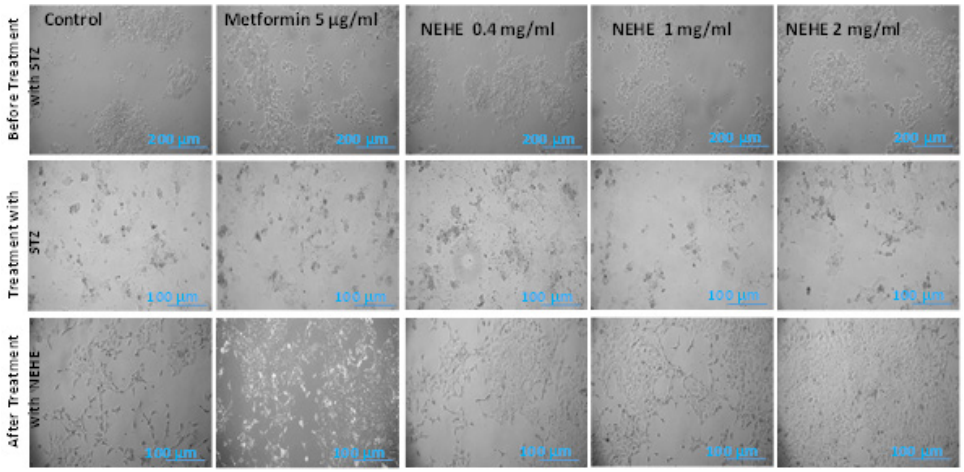

Fig. 2. Morphology of RIN-5F cells before STZ, after STZ, and after NEHE treatments. After treatment with the NEHE, the cells were spindle-shaped and had normal morphology

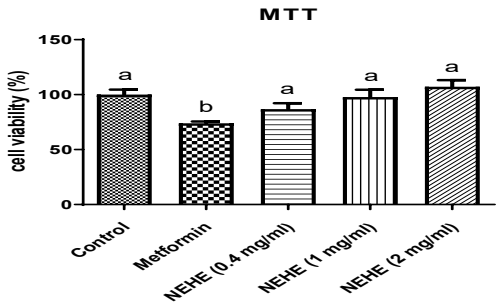

Fig. 3. MTT assay to determine the survival percentage of cells after treatment with either metformin or different concentrations of NEHE. The different alphabetic signs on each column represent statistically significant differences between the mean values (P<0.05), and the same signs are insignificant

survival rate increased (Fig. 3).

Examining the level of insulin expression by immunohistochemistry test

This test determines the level of insulin expression by cells after different treatments [23]. Diabetic cells treated with metformin showed that the level of insulin expression was significantly lower (P<0.001) than that of the control group (healthy RIN-5F cells), indicating that this antidiabetic drug could not alter the insulin expression to the level of the control group. After a 72 hr treatment with NEHE (0.4 mg/ml), insulin expression is significantly lower (P<0.001) than cells in control and metformin groups. In the case of NEHE (1 mg/ml), although insulin expression increased, but it was significantly lower (P<0.001) than those of the control and metformin groups. However, NEHE (2 mg/ml) increased insulin expression significantly (P<0.01) compared to that of the metformin group. With the increase in NEHE concentration, the level of insulin expression increased gradually (Fig. 4).

Examining the expression of ϐGK and GLUT2 genes by the qRT-PCR method

The expression of genes involved in diabetes was investigated at the mRNA level to determine the antidiabetic effect of NEHE. BGK gene expression in diabetic RIN-5F cells increased significantly after treatment with metformin compared to the diabetic control group (P<0.001). In extract treatment groups, diabetic cells were treated with various concentrations of NEHE for 72 hr. The expression of BGK was not significantly different from the control group at a concentration of 0.4 mg/ml, but NEHE1 at 2 mg/ml, increased the expression significantly compared to the control group, consecutively (P<0.05) and (P<0.001). The level of BGK gene expression in the NEHE (2 mg/ml) was not significantly different from that of the metformin group (Fig. 5a).

The expression of the GLUT2 gene in cells treated with metformin increased significantly compared to the diabetic control group (P<0.001). After 72 hr treatment of diabetic cells with different concentrations of NEHE, the expression of GLUT2 at the 0.4 and 1 mg/ml concentrations was similar to the control group and had no significant difference. However, NEHE)2 mg/ml) significantly increased the gene expression level compared to the control group (P<0.01). Although the expression of the GLUT2 gene increased by using NEHE (2 mg/ml), the level of expression could not reach the level of the metformin group (P<0.01) (Fig. 5b).

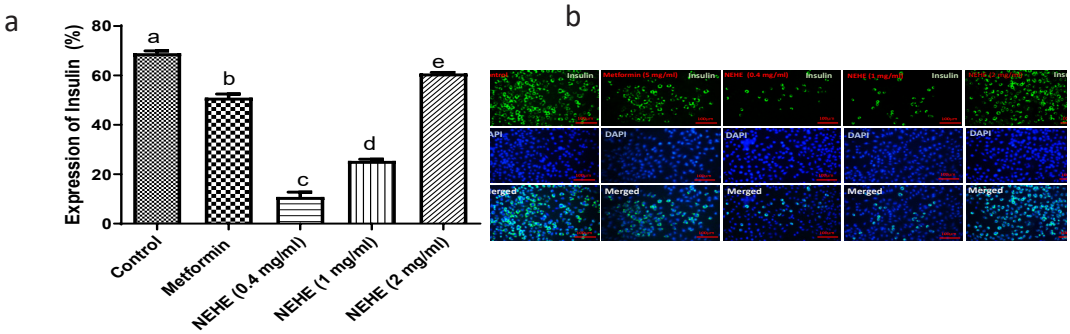

Fig. 4. The data shows the percentage of insulin expression in different treatment groups. Similar letters indicate the absence of significant differences between groups, while different letters indicate significant differences between groups (a). Immunohistochemistry of insulin expression in control, metformin (5 mg/ml), and NEHE (0.4, 1 and 2 mg/ml) groups. In the first row, the expression of insulin is indicated by green fluorescent color. In the second row, the nuclei of the cells are shown in blue, which indicates the cells. In the third row, a combination of two colors is displayed to clarify the cell nucleus and the insulin expression level (b)

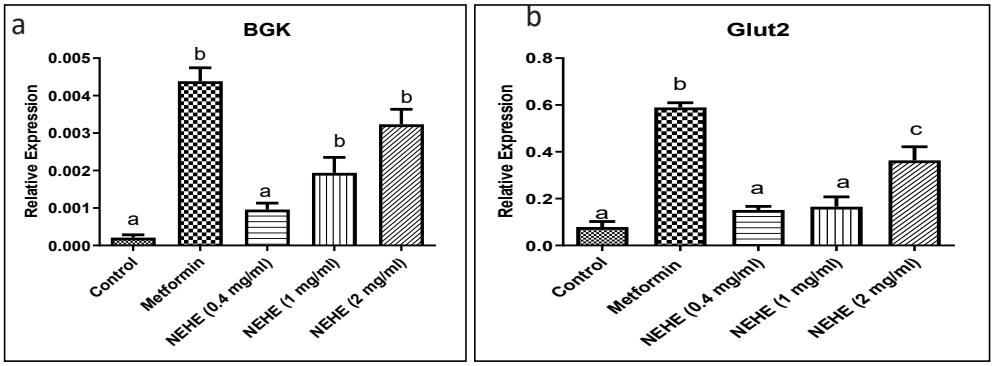

Fig. 5. The expression levels of genes in RIN-5F cells after induction of diabetes using streptozotocin and after 72 hr of treatment in different groups. a) The expression of the BGK gene in the metformin and NEHE (1 and 2 mg/ml) groups shows a significant increase compared to the control group, respectively (P<0.05) and (P<0.001). b) GLUT2 gene expression in the metformin group and NEHE 2 mg/ml shows a significant increase compared to the control group (P<0.01). Similar letters indicate the absence of significant differences between groups, while different letters indicate significant differences between groups

Examining the gene expression of PPARγ and MiR-27a using the qRT-PCR method

PPARG gene closely interacts with MiR-27a in increasing insulin sensitivity. The expression of PPARG and miR-27a genes was investigated to determine the antidiabetic effect of NEHE (Fig. 6). The expression of PPARγ in cells treated with metformin increased significantly (P<0.001) compared to that of the control group (diabetic treated with streptozotocin). After 72 hr treatment of diabetic cells with different concentrations of NEHE, the expression of PPARγ in NEHE (0.4 mg/ml) was not significantly different from that of the control group. The gene expression level increased significantly at 1 and 2 mg/ml concentrations of extract compared to that of the control group (P<0.001). Furthermore, at these two concentrations of NEHE, PPARγ gene expression level increased significantly as much as the metformin group.

The expression of mir-27a in cells treated with metformin increased significantly compared to the control group (diabetic treated with streptozotocin) (P<0.001). After 72 hr treatment of diabetic cells with different concentrations of NEHE, the expression of miR-27a at a concentration of 0.4 mg/ml was similar to the control group and had no significant differences. However, its expression increased significantly after the cell treatment with NEHE at concentrations of 1 and 2 mg/ml, consecutively (P<0.05) and (P<0.01).

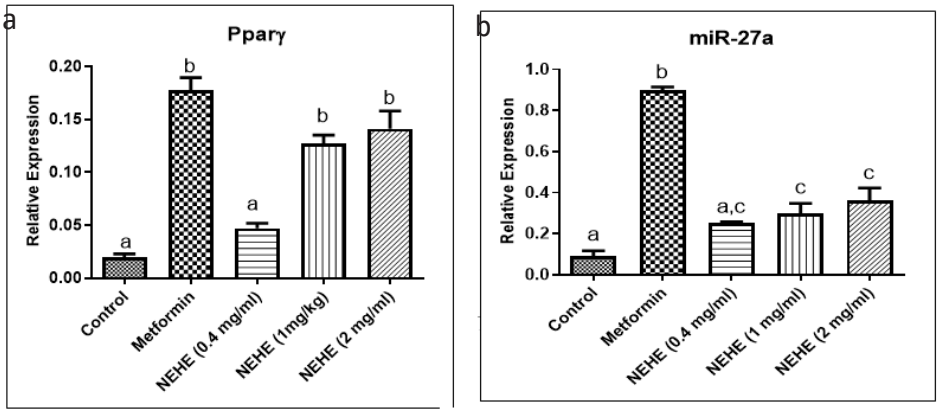

Fig. 6. PPARG and MiR-27a gene expression level in RIN-5F cells after induction of diabetes using streptozotocin and 72 hr treatment in different groups. a) The expression of PPARG in NEHE (1 and 2 mg/ml) groups was not significantly different from the metformin group. b) The expression of MiR-27a in the metformin and NEHE (1 and 2 mg/ml) groups show a significant increase compared to the control group (P<0.01). Similar letters indicate the absence of significant differences between groups, while different letters indicate significant differences between groups

DISCUSSION

Diabetes is a common metabolic disorder affecting more than 170 million people yearly [24]. This disease is characterized by chronic hyperglycemia and increases the risk of stroke, cardiovascular and kidney disease as the most common complications of this disease. [4].

This study showed the antidiabetic effect of NEHE on RIN-5F cells through increased insulin secretion from these cells. Moreover, NEHE at the dose of 2 mg/ml increased the expression of genes involved in diabetes, probably through the miR-27a pathway.

GLUT2 is a glucose transporter and controls the balance between the concentration of glucose inside and outside the cell and also known as a glucose sensor [7, 24]. BGK plays an essential role in glucose homeostasis, and insulin plays an important role in regulating blood glucose [25]. Thus, green tea which contains flavonoids, can exert insulin-like and insulin-enhancing activities, thus affecting the BGK function [12]. In addition, one of the functions of chicory is blood sugar control [13]. Garlic and diallyl trisulfide, present in garlic, increase the tolerance of oral glucose and insulin secretion and activity [14]. All these factors help BGK to regulate blood glucose in diabetic people properly [6]. PPARy is not a metformin agonist but acts like it in diabetes 2. When activated, reduces hyperglycemia by increasing peripheral insulin sensitivity, reducing glucose production, and regulating insulin sensitivity [8].

An increase in the level of free fatty acids in blood circulation and fat accumulation in non-fat tissues play a role in insulin resistance [9]. Green tea has a phenolic antioxidant called epigallocatechin gallate (EGCG). EGCG, like PPARy, decreases the number of fat cells and fat precursor cells and ultimately reduces insulin resistance. Catechin in green tea reduces the effects of insulin resistance through the digestion and absorption of carbohydrates [26]. It has been reported that this antioxidant reduces the amount of glucose in the liver cells of mice, mimics insulin hormone, and increases tyrosine phosphorylation of the insulin receptor and the insulin receptor substrate [26-28]. Moreover, studies have shown that the blood glucose level is reduced more effectively by garlic extract compared to glibenclamide (an antidiabetic medication) in diabetic rats [29].

In the early stages of diabetes treatment, the chicory plant can increase insulin levels and sensitivity [30]. Chicory can also reduce insulin resistance. Oral administration of garlic extract reduces the amount of FBG (Fasting Blood Sugar), cholesterol, triglyceride, urea, uric acid, and liver enzymes AST and ALT. Therefore, insulin secretion increases, cholesterol and fatty acid synthesis are inhibited, thereby reducing insulin resistance [13]. When blood glucose levels rise, miR-27a expression is stimulated, and PPARy expression is inhibited. MiR-27a inhibits PPARy expression in insulin-resistant cells [31]. When glucose is low, miR-27a interacts with PPARy to increase insulin sensitivity [22]. High expression of miR-27a reduces glucose uptake and consumption [9]. When induced insulin secretion by glucose is impaired in diabetes, garlic can increase the half-life of insulin [32], while green tea and chicory reduce insulin resistance through miR-27a [9].

CONCLUSION

The data of this study showed that NEHE, including green tea, chicory, and garlic, was not toxic to rat beta cells. In the NEHE (2 mg/ml) group, insulin expression was significantly higher than that of the metformin group, which indicates the antidiabetic effect of NEHE. In terms of the expression of genes involved in diabetes, the expression of PPARy and BGK genes in cells treated with NEHE (2 mg/ml) did not differ significantly compared to that of the metformin group, indicating that the therapeutic effect of NEHE is equivalent to the standard antidiabetic drug metformin. Furthermore, regarding the expression of GLUT2 and miR-27a genes, although they were lower in the NEHE group than in the metformin group, they were significantly increased compared to the control group. Overall, the results of this study indicated the anti-diabetic effect of the NEHE.

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CONFLICTS OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

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