Increased Glucose Uptake and Insulin Binding Activity of *Nerium Oleander* in Hepatocytes and Adipocytes [1]

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Summary

Type 2 diabetes mellitus (DM) affects a large population worldwide. DM is often considered as a syndrome of disordered glucose metabolism. Current conventional drug therapies for DM are usually insufficient and medicinal herbs with antihyperglycemic activities are increasingly sought by diabetic patients and health care professionals. *Nerium Oleander* (N.O.) is known to be effective in lowering of postprandial blood glucose in DM patients as a folk remedy. In this study we aimed to evaluate effect of N.O. distillate in glucose uptake activity of hepatocytes and adipocytes. The human hepatoma cells Hep3B and mouse adipocyte 3T3-L1 cells were cultured. Depending on the groups, different concentrations insulin (1, 10, 20 IU/ml) and N.O. (0.1, 1, 10, 50 µg/ml) were added to medium for 48 h. Cellular toxicity and proliferation were evaluated by LDH secretion levels and MTT test. A metabolizable fluorescent derivative of glucose, 2-NBDG and FITC-insulin were used for glucose uptake and insulin binding activity. Insulin increased cellular proliferation and decreased LDH leakage and apoptosis in both cell types. Lower dosages of N.O. has no significant effect on apoptosis and cell number while at the highest dosages minimal cytotoxicity was seen mainly in adipocytes. Main effect of N.O. treatment was increased glucose uptake in Hep3B and 3T3-L1 cells (P<0.001). Our results showed that N.O. may be offered as new approaches to treatment of type 2 diabetes by modulating cellular glucose uptake.

*Keywords*: Nerium oleander, Hepatocytes, Adipocytes, Glucose uptake, Insulin binding, Type II diabetes mellitus

*Nerium Oleander*’in Hepatositler ve Adipositlerde İnsulin Bağlanması ve Glukoz Alımı Artrtıcı Etkisi

Özet


Anahtar sözcükler: Nerium oleander, Hepatositler, Adipositler, Glukoz alımı, Insülin bağlanması, Tip II Diabetes mellitus

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INTRODUCTION

Type 2 diabetes mellitus (DM) affects a large population worldwide. There have been many attempts to develop the safe and effective methods of curing diabetes. Although very intensive research is being conducted in this field, current protocols still have only limited applications. The general consensus on treatment of type 2 diabetes is that lifestyle management is at the forefront of therapy options. In addition to exercise, weight control, and medical nutrition therapy, oral glucose-lowering drugs and injections of insulin are the conventional therapies. Alternative treatments for diabetes have become increasingly popular the last several years, including medicinal herbs, nutritional supplementation and acupuncture. Plant derivatives with purported hypoglycemic properties have been used in folk medicine and traditional healing systems around the world. Approximately 1200 plants are used worldwide for the empirical treatment of DM. However, only about 350 of them are documented to present hypoglycemic activity although only a small number of these have received scientific and medical evaluation to assess their efficacy.

The World Health Organization Expert Committee on diabetes has recommended that traditional medicinal herbs be further investigated.

Some of the most common plants used traditionally for treatment of diabetes are Pterocarpus marsupium, Bitter Melon, Gymnema Sylvestre, Asian Ginseng, Soybean and Cinnamon.

*Nerium oleander* (N.O.) is a member of Apocynaceae familia and grown in Mediterranean region. *Nerium indicum* is known to be effective in lowering of postprandial blood glucose in DM patients and both oleander and indicum sub-forms are now used as a folk remedy for type II diabetes in some regions of mediterranean region and Asia. The hot water extract of the leaves of N.O. has been used as a remedy against Type II DM with subjective success but without corroborating laboratory data. First report related to the hypoglycemic activity of the plant is given by Bellakhdar et al. and then reported by other authors.

DM is a complex group of metabolic disorders including hyperglycemia and impaired insulin secretion and/or insulin response. Current theories of DM include a defect in insulin-mediated glucose uptake in muscle and adipocytes, a dysfunction of the pancreatic β-cells, an impaired insulin action in liver and decreased peripheral (muscle) glucose utilization. Changes in glucose clearance, an index of efficiency of glucose removal from the circulation, by itself do not affect plasma glucose concentrations independent of changes in rates of glucose entry and exit. In this study we aimed to evaluate in vitro direct effect of N.O. in hepatocyte and adipocyte cells especially with the aspect of glucose uptake metabolism and cellular proliferation.

MATERIAL and METHODS

**Cell lines, Chemicals and Materials**

Human hepatoma cell line Hep3B and mouse preadipocyte cells 3T3-L1 were obtained from the American Type Culture Collection (ATCC). Hep3B cells were cultured in Roswell Park Memorial Institute-1640 (RPMI, PAA, Austria) and 3T3-L1 cells in Dulbecco’s Modified Eagles Medium (DMEM, PAA, Austria), supplemented with fetal calf serum (FCS), (PAA, Austria), L-glutamine, streptomycin and penicillin (Sigma, MO, USA). Insulin (Sigma, MO, USA) was dissolved in water, sterilized by 0.22 m pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations. Cell counts were tested by 3-[4,5-di methyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, MO, USA). Lactate Dehydrogenase (LDH) and glucose levels were measured with commercial kits using an automatic multi-analyzer (Roche; P800).

**Obtaining Liyophilized Nerium oleander**

*Nerium oleander* plant was collected among new shoots in March-September period from Mediterranean region of Turkey. After washing collected plant, fresh shoots were chopped, and adequate distilled water added. The mixture was heated in heat resistant container. After liquid started to evaporate, container lid was covered and vapor was separated to other clean glass container by causing it to come in contact with a surface cooled with cold water. N.O. distillate was liyophilized in small glass bottle (20 ml) by using liyophilizator. N.O. liyophilized distillate was dissolved in saline solution then sterilized by 0.22 m pore size cellulose acetate membrane filters, and added to cultures at the indicated time and concentrations.

**Cell Culture and Experimental Protocol**

The human hepatoma cell line Hep3B was cultured in RPMI-1640 medium and 3T3-L1 cells in DMEM, supplemented with 10% v/v fetal calf serum, 2 mmol/L L-glutamine, streptomycin (100 mg/mL) and penicillin (100 IU/mL) in a humidified atmosphere containing 5% CO₂ at 37°C. One day before the experiments, cells were seeded on 96-well microtitre plates (Nunc, Denmark) at 2X10³ cells/mL.

Depending on the groups, different concentrations of insulin (1, 10, 20 IU/ml) and N.O. (0.1, 1, 10, 50 µg/ml) were added to medium for 48 h.

LDH levels were evaluated from control and treated cells at the 48th h. MTT was measured at the 2nd, 24th and 48th h. After supernatants were removed cell surface was washed with sterile phosphate buffered saline (PBS) and cells were harvested with lysis solution and caspase-3 levels of groups were measured from cell lysates. LDH measurement was done from both of the supernatant and cell lysates.
Evaluation of Cellular Proliferation or Death

MTT, a colorimetric assay based upon the ability of living cells to reduce MTT into formazan, was used for evaluation of the effects of dose and time dependent effects of glucose, insulin and N.O. on cellular death or proliferation (24th, 48th h). Cell number % was calculated as ratio of cell number of effected group vs control group 100 at the determined hours.

Biochemical Determination of Cell Death

Hep3B cells were plated in 96 multiwell cell culture plates as 3x10³ cells/mL. LDH is normally present in the cytosol of hepatocytes. In response to cell damage LDH is released from the cells. Therefore, to determine cell death, we measured secreted and intracellular LDH levels and calculated % released LDH at the 48th h for each group. To do this, the medium was collected to measure enzyme activities. The adherent cells were lysed. Both medium and cell lysates were used for quantitative determination of LDH activity (IU/L) which was performed with an automatic multianalyzer (Roche, MN, USA) using commercial kit (Roche, MN, USA). Released enzyme fractions for each sample were calculated as the ratio of enzyme present in the medium vs the sum of the levels of same enzyme in the supernatant and in the cells.

Glucose Uptake and Insulin Binding Activity

A metabolizable fluorescent derivative of glucose, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Molecular Probes, Oregon, USA), was used. Following incubation at 37°C for 120 min with the dye, culture medium was removed and after washing the cells with Hanks' balanced salt solution (HBSS) fluorescence was measured in Spectromax M2 fluorescence microplate reader (Perkin-Elmer Corp. Norwalk, CT, USA) set at an excitation wavelength of 470 nm and an emission wavelength of 520 nm. To determine the effect of insulin or N.O. on glucose uptake, the cell suspension was dispensed in 96-well microtiter plates at 2500 cells/well (200 µL). After 24 h of incubation (37°C/ 5% CO₂), all culture medium was removed from each well and replaced with 100 µL of culture medium with different concentrations of insulin and and 100 µL of 300 µM 2-NBDG diluted in the same medium to give a final concentration of 150 µM /well. In this case, cells were incubated simultaneously with both insulin/N.O. and 2-NBDG for 120 min, and then the plates were centrifuged, the culture medium was removed and the cells were washed once with HBSS. Fluorescence was measured at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Statistical Analysis

Results of the experiments were analyzed by One Way ANOVA, followed by a multiple comparison test using SPSS 13.0. P<0.05 was accepted as statistically significant. Results were given as mean± SEM.

RESULTS

We characterized the concentration-dependent effect of N.O. and insulin on human hepatocyte cell line (Hep3B) and mouse adipocyte cell line (3T3-L1) as a function of time. N.O. treatment decreased adipocyte cell number (P<0.01) whereas insulin increased (P<0.01, Fig. 1). Minimal toxic effect is seen at the highest dose of N.O. (P<0.05) in Hep3B cells (Fig. 2).

Insulin treatment decreased LDH leakage, higher doses of N.O. treatment increased LDH leakage (P<0.01, Fig. 3).

Main effect of N.O. treatment was seen in glucose uptake to Hep3B and 3T3-L1 cells (P<0.001, Fig. 4, 5) and this effect is more prominent in liver cells and significantly different from control and insulin treated cells (P<0.001). N.O. treatment increased insulin binding capacity in both adipocytes and hepatocytes (P<0.001, Fig. 6).

DISCUSSION

Both experimental and epidemiologic studies showed that insulin resistance is not key factor only in diabetes also in cardiovascular diseases 19. Control of blood glucose levels is a function and coordination of different organ systems such as liver, pancreas, muscle and fat. These organs and tissues

Fig 1. Cell number was determined at 24th and 48th h in adipocyte cell line 3T3-L1. NO treatment caused significant cytotoxicity with dose and time dependent manner (P<0.01). The effect is most prominent in N.O. 10 µg/ml dose and at 24 h (P<0.001). Insulin treatment increased cell number at 48 h in all concentrations (P<0.01). Data are presented as mean±SEM (n=6).

Şekil 1. Adiposit hücre dizisinde (3T3-L1) hücre proliferasyonunun 24 ve 48. saatlerde değerlendirilmesi. N.O. tedavisi doz ve zaman bağımlılı olarak sítotoksik etki göstermiştir (P<0.01). Bu etki NO 10 µg/ml dozda ve 24. saatte en belirgindir (P<0.001). Insulin tedavisi 48. saatte hücre proliferasyonunu tüm dozlarda artırılmıştır (P<0.01). Sonuçlar ortalama±SEM olarak verilmiştir (n=6)
have major roles in the use and storage of nutrients in the form of glycogen and triglycerides. Type II DM is the most common disorder which is characterized by impaired insulin stimulated glucose uptake in muscle and adipose tissue. It is striking that peripheral glucose levels should be well regulated by physiological control mechanisms, which, when deregulated, trigger early signs of the pathogenesis of obesity and diabetes, such as abnormal suppression of glucagon and loss of insulin secretion in the fed state and decreased peripheral tissue glucose uptake. These dysregulations become prominent before full impairment...
of beta-cell secretion and insulin resistance appears, a characteristic of established type 2 DM. Also dysregulation of extrapancreatic glucose sensors, especially glucose uptake, may be early events in the pathogenesis of obesity and type 2 diabetes. From the physiologic standpoint, activation of glucose transport and glycogen synthase is linked to the insulin-signaling mechanism in many systems. Glucose entry into the primary insulin target tissues (skeletal muscle, heart, adipose tissue, and liver) occurs by facilitated diffusion, mediated by a family of transport proteins. Glucose transporters (GLUT) mainly GLUT 1-4 mediates insulin stimulated glucose uptake by skeletal muscle, heart, and adipose tissues. Adipocyte metabolism starts to take famous roles in recent studies. It is accepted now that altered adipocyte metabolism, fat storage and distribution is very important in the pathogenesis of glucose intolerance in Type 2 DM. Chronically increased glucose and plasma free fatty acids FFA stimulate adipogenesis and further glucose-neogenesis leading to hepatic/muscle insulin resistance. Dysfunctional fat cells produce excessive amounts of insulin resistance-inducing, inflammatory, and atherosclerotic-provoking cytokines and fail to secrete normal amounts of insulin-sensitizing adipocytokines. Fat cells start to proliferate and become enlarged and more insulin resistant. Different therapeutics are used to enhance adipocyte insulin sensitivity, reduce plasma FFA, and favorably influence the production of adipocytokines. Thiazolidinediones are insulin-sensitizing antiabetic agents which are used in DM patients. Researchers are seeking new alternative treatments to decrease intracellular concentrations of triglyceride metabolites in muscle, liver, and B-cells, contributing to improvements in muscle/hepatic insulin sensitivity and pancreatic and liver function in type 2 diabetics.

Alternative therapies with anti-hyperglycemic effects are increasingly sought by patients with diabetes. This comes as no surprise since alternative treatments have been most widely used in chronic diseases, which may be only partially alleviated by conventional treatment. Herbal medications are the most commonly used alternative therapy for blood sugar control; however, their safety and efficacy need to be further evaluated by well-designed, controlled clinical studies. N.O. is well known toxic plant especially with its chemotherapeutic potential. Yassin and Mwafy showed anti-lipidemic effect without toxicity at the dose Haeba et al. determined with toxicological studies of N.O. leaflets. Similarly; Gayathri et al. found that N.O. extract had an antilipidemic effect in high fat diet fed rats.

Hepatotoxicity of N.O. extract is tested by MTT, LDH leakage measurements in human hepatocytes and we did not find toxic effect or minimal toxicity is found with higher dosages. However, the action mechanism of N.O. is not known yet in the regulation of glucose metabolism. We found that N.O. increases glucose uptake in both cell type, especially in liver cells which is more important for glucose retrieval from blood. Our data suggest that N.O. acts through increase in insulin binding and may effect glucose utilization in adipocytes, and hepatocytes.

In summary, we conclude that N.O. is a bioactive component in hepatocytes and adipocytes and able to regulate glucose metabolism and insulin sensitivity in cells. Further investigations are required to evaluate the underlying mechanisms of benefit of N.O. treatment.

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REFERENCES

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