DOI: 10.2478/10004-1254-60-2009-1917

Scientific Paper

COMPARATIVE EFFECTS OF CALCIUM CHANNEL BLOCKERS, AUTONOMIC NERVOUS SYSTEM BLOCKERS, AND FREE RADICAL SCAVENGERS ON DIAZINON-INDUCED HYPOSECRETION OF INSULIN FROM ISOLATED ISLETS OF LANGERHANS IN RATS*

Nazila POURKHALILI[1], Shirin POURNOURMOHAMMADI[2], Fatemeh RAHIMI[3], Sanaz VOSOUGH-GHANBARI[3], Maryam BAEERI[3], Seyed Nasser OSTAD[3], and Mohammad ABDOLLAHI[3]

Pharmaceutical Sciences Branch, Islamic Azad University, Tehran[1]; Institute of Medicinal Plants, ACECR, Tehran[2]; Faculty of Pharmacy, and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran[3]

Received in September 2008
Accepted in February 2009

Hyperglycaemia has been observed with exposure to organophosphate insecticides. This study was designed to compare the effects of calcium channel blockers, alpha-adrenergic, beta-adrenergic, and muscarinic receptor blockers, and of free radical scavengers on insulin secretion from diazinon-treated islets of Langerhans isolated from the pancreas of rats using standard collagenase digestion, separation by centrifugation, and hand-picking technique. The islets were then cultured in an incubator at 37 °C and 5 % CO_2. In each experimental set 1 mL of 8 mmol L^{-1} glucose plus 125 μg mL^{-1} or 625 μg mL^{-1} of diazinon were added, except for the control group, which received 8 mmol L^{-1} glucose alone. The cultures were then treated with one of the following: 30 μmol L^{-1} atropine, 100 μmol L^{-1} ACh + 10 μmol L^{-1} neostigmine, 0.1 μmol L^{-1} propranolol, 2 μmol L^{-1} nifedipine, 50 μmol L^{-1} phenoxybenzamine, or 10 μmol L^{-1} alpha-tocopherol. In all experiments, diazinon significantly reduced glucose-stimulated insulin secretion at both doses, showing no dose dependency, as the average inhibition for the lower dose was 62.20 % and for the higher dose 64.38 %. Acetylcholine and alpha-tocopherol restored, whereas atropine potentiated diazinon-induced hyposecretion of insulin. Alpha-, beta- and calcium channel blockers did not change diazinon-induced effects. These findings suggest that diazinon affects insulin secretion mainly by disturbing the balance between free radicals and antioxidants in the islets of Langerhans and by inducing toxic stress.

KEY WORDS: *organophosphates, oxidative stress, pancreas, rats*

* This study has been presented at EUROTOX 2008 and the abstract published in a Toxicology Letters supplement.

Diazinon {O,O-diethyl-O-[6-methyl-2- (1-methylethyl)-4pyrimidinyl] phosphorothioate} is a widely used organophosphorus (OP) insecticide. It may pollute the environment, enter the food cycle, and cause chronic toxic effects in humans. The main mechanism of OP action is to inhibit acetylcholinesterase (AChE) activity in target tissue (1). Poisoning with OPs is a major health concern that has not been resolved (2-7). In addition to cholinergic effects usually observed with OPs, Rahimi and Abdollahi (8) singled out hyperglycaemia as one of the consequences of both acute and chronic exposure to OPs. Among different mechanisms that have been suggested for OP-induced hyperglycaemia, effects on pancreatic islets have received much attention in recent years. Research on islets of Langerhans isolated from rats pre-treated subchronically with malathion has indicated that insulin secretion is inhibited in the presence of basal and stimulatory concentrations of glucose (9-11).

Our earlier studies have shown that subchronic exposure of rats to malathion increased both blood glucose and insulin and muscle phosphofructokinase and glycogen phosphorylase (GP) activities, resulting with increased glycogenolysis and glycolysis (12). It has also been found that malathion stimulates GP and phosphoenolpyruvate carboxykinase (PEPCK) activities in the liver (13). On the other hand, there is evidence that glycogen may be stored in the liver due to higher insulin secretion after the inhibition of AChE activity in pancreatic B-cells by subchronic administration of malathion (14). In addition, administration of diazinon to rats increased serum glucose and decreased glycogen content in the brain while glycogenolytic activities of glycogen phosphorylase and phosphoglucomutase increased significantly (15).

Furthermore, antioxidants like alpha-tocopherol and N-acetylcysteine (NAC) have shown a protective role against OP-induced glucose changes (6, 16). There is also evidence that the efficiency of phosphodiesterase inhibitors in countering diazinon-induced hyperglycaemia depends on their antioxidant potential (17, 18).

Insulin secretion from the islets of Langerhans is influenced by muscarinic cholinergic and then adrenergic systems. Calcium channels are also involved in mediating the effects of neurotransmitters (19). This study was designed to compare the effects of calcium channel blockers, alpha-adrenergic, beta-adrenergic, and muscarinic receptor blockers, and free radical scavengers on insulin secretion from isolated rat islets of Langerhans in the presence of diazinon.

MATERIALS AND METHODS

Chemicals

2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), collagenase V, NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $Na_2HPO_4 \cdot 12H_2O$, KH_2PO_4, $NaHCO_3$, glucosexH_2O, $CaCl_2 \cdot 2H_2O$, NaH_2PO_4, $MgCl_2$, HCl, bovine serum albumin (BSA), alpha-tocopherol (Trolox®), acetylcholine, neostigmine, and diazinon were purchased from Sigma-Aldrich Co. (Dorset, England). RPMI medium and its supplements were purchased from Invitrogen Co. (Gibco, UK). Rat insulin ELISA kit was purchased from Mercodia Co. (Uppsala, Sweden). Propranolol, nifedipine, phenoxybenzamine, and atropine were obtained from local pharmaceutical companies.

Animals

Male Wistar rats weighing 200 g to 250 g were housed in polypropylene cages under standard conditions with free access to drinking water and food, 12-h light : 12-h dark cycle, and an ambient temperature of (20 to 25) °C. All experiments were performed according to the Animal Welfare Act, and the study protocol was approved by the ethics committee of the Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences.

Islet isolation and culture

Rats were anaesthetised with intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1}) and underwent laparatomy; the common bile duct was ligated at its exit into the liver. The duct was then cannulated at its exit from the duodenum. Then the pancreas was distended by injecting 10 mL of cold collagenase V (1 mg mL^{-1}) prepared in Hanks-HEPES buffer (8 g L^{-1} NaCl, 0.4 g L^{-1} KCl, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 0.06 g L^{-1} KH_2PO_4, 0.35 g L^{-1} $NaHCO_3$, 2.32 g L^{-1} Hepes, 0.4 g L^{-1} glucose $\cdot H_2O$, 0.186 g L^{-1} $CaCl_2 \cdot 2H_2O$, pH 7.2). After perfusion, the islets were kept in Krebs buffer (8 g L^{-1} NaCl, 0.27 g L^{-1} KCl, 0.42 g L^{-1} $NaHCO_3$, 0.06 g L^{-1} NaH_2PO_4, 0.05 g L^{-1} $MgCl_2$, 2.38 g L^{-1} HEPES, 0.22 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.5 g L^{-1} glucose $\cdot H_2O$, pH 7.4), centrifuged, separated from the remaining tissue by hand-picking under a stereomicroscope, and incubated

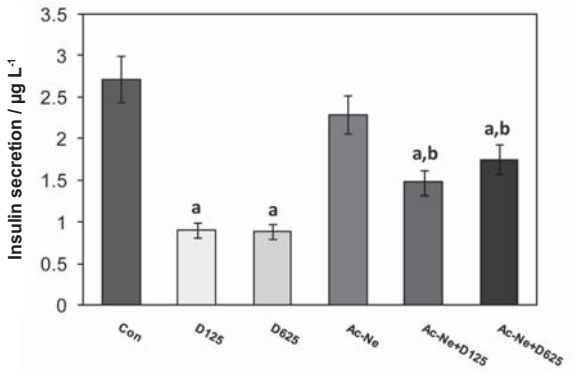

Figure 1 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon (D) alone or in combination with acetylcholine (Ac) and neostigmine (Ne). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 100 µmol L^{-1} acetylcholine and 10 µmol L^{-1} neostigmine alone, or diazinon in combination with 100 µmol L^{-1} acetylcholine and 10 µmol L^{-1} neostigmine were used. Values are means±SEM. [a] P<0.05 for difference from control, [b] P<0.05 for difference from diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}.*

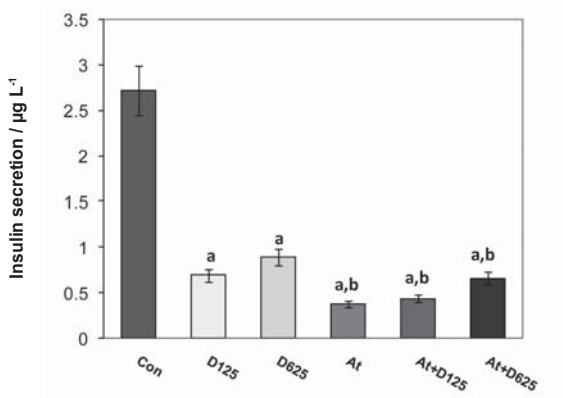

Figure 2 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon (D) alone or in combination with atropin (At). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 30 mmol L^{-1} atropine alone, or diazinon in combination with 30 mmol L^{-1} atropine were used. Values are means±SEM. [a] P<0.05 for difference from control.*

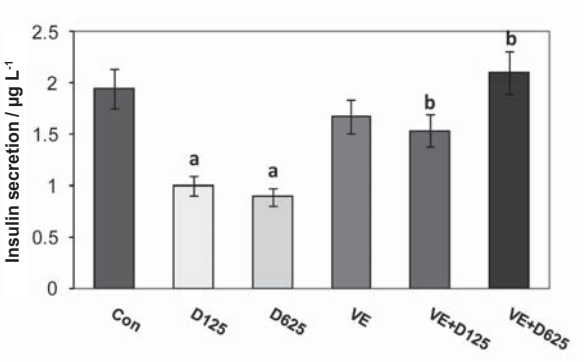

Figure 3 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon (D) alone or in combination with alpha-tocopherol (VE). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 10 µmol L^{-1} alpha-tocopherol alone, or diazinon in combination with 10 µmol L^{-1} alpha-tocopherol were used. Values are means±SEM. [a] P<0.05 for difference from control, [b] P<0.05 for difference from diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}.*

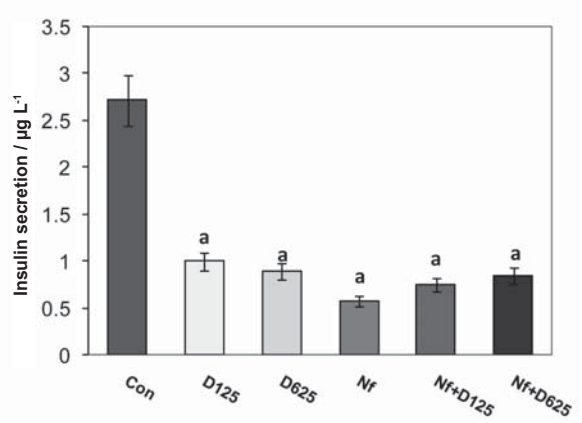

Figure 4 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon (D) alone or in combination with nifedipine (Nf). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 2 µmol L^{-1} nifedipine alone, or diazinon in combination with 2 µmol L^{-1} nifedipine were used. Values are means±SEM. [a] P<0.05 for difference from control, [b] P<0.05 for difference from diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}.*

overnight in a supplemented RPMI-1640 medium containing 10 % BSA, 1 % penicillin-streptomycin, and 0.1 % gentamycin at 5 % CO_2 and 37 °C (10).

Static insulin secretion

The islets were washed in Krebs-Ringer buffer and then pre-incubated for 30 min in a water bath with the same buffer at 37 °C. The islets were washed again and then dispensed in batches of 10 using a stereomicroscope. In each experimental set 1 mL of 8 mmol L^{-1} glucose plus 125 µg mL^{-1} or 625 µg mL^{-1} of diazinon were added except for the control group, which was treated with 8 mmol L^{-1} glucose alone. Batches of 10 islets were then treated

with one of the following solutions: 30 µmol L^{-1} atropine, 100 µmol L^{-1} ACh+10 µmol L^{-1} neostigmine, 0.1 µmol L^{-1} propranolol, 2 µmol L^{-1} nifedipine, 50 µmol L^{-1} phenoxybenzamine, or 10 µmol L^{-1} alpha-tocopherol. The low and the high dose of diazinon were around 1/10 and 1/3 of its LD_{50}, respectively (9-11). ACh degradation was prevented by neostigmine, as it inhibits AChE activity.

The islets were incubated for 30 minutes in a water bath (at 37ºC), tubes were placed on ice, and the supernatant was taken to measure secreted insulin using the ELISA method (10).

Statistical analysis

Data were expressed as means ± SEM of separated experiments and analysed using one-way ANOVA followed by Dunnett post hoc multiple comparison tests. The significance level was set at $P<0.05$.

RESULTS

Glucose-stimulated insulin secretion

In all experiments, either dose of diazinon significantly ($P<0.05$) reduced glucose-stimulated insulin secretion. The average inhibition was 62.20 % at 125 µg mL^{-1} and 64.38 % at 625 µg mL^{-1}, and showed no dose-dependence (Figures 1-6). ACh+neostigmine with 125 µg mL^{-1} diazinon increased glucose-stimulated insulin secretion 64.4 % in comparison with diazinon given alone while ACh+neostigmine with 625 µg mL^{-1} diazinon increased it 95.5 % in comparison with diazinon given alone (Figure 1). Atropine alone decreased glucose-stimulated insulin secretion to 13.98 % of control. Atropine plus diazinon at either dose further reduced insulin secretion compared to diazinon alone (Figure 2). Alpha-tocopherol plus 125 µg mL^{-1} diazinon increased glucose-stimulated insulin secretion 54 % in comparison with diazinon given alone while alpha-tocopherol plus 625 µg mL^{-1} diazinon increased it 134 % in comparison with diazinon given alone (Figure 3). Nifedipine decreased glucose-stimulated insulin secretion to 21.4 % of control. Nifedipine plus diazinon did not alter insulin secretion in comparison with diazinon alone (Figure 4). Phenoxybenzamine plus diazinon did not alter insulin secretion in comparison with diazinon alone (Figure 5).

Propranolol decreased glucose-stimulated insulin secretion to 21.7 % of control. Propranolol plus diazinon did not alter insulin secretion in comparison with diazinon alone (Figure 6).

DISCUSSION

The results of this study have shown that diazinon significantly reduces glucose-stimulated insulin secretion in a manner that is not dose-dependent (62.20 % inhibition at 125 µg mL^{-1} and 64.38 % at 625 µg mL^{-1}). Among the blockers tested, only

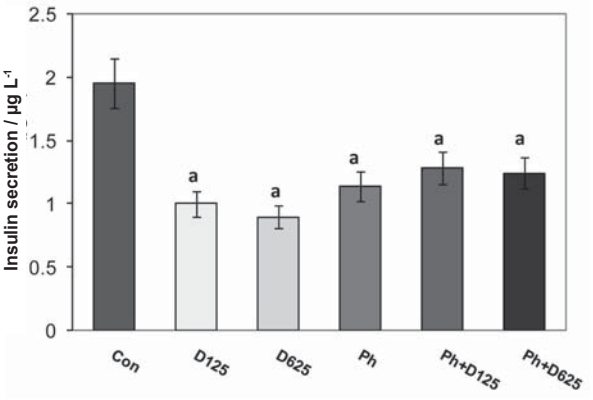

Figure 5 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon (D) alone or in combination with phenoxybenzamine (Ph). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 50 µmol L^{-1} phenoxybenzamine alone, or diazinon in combination with 50 µmol L^{-1} phenoxybenzamine were used. Values are means±SEM. [a] $P<0.05$ for difference from control.*

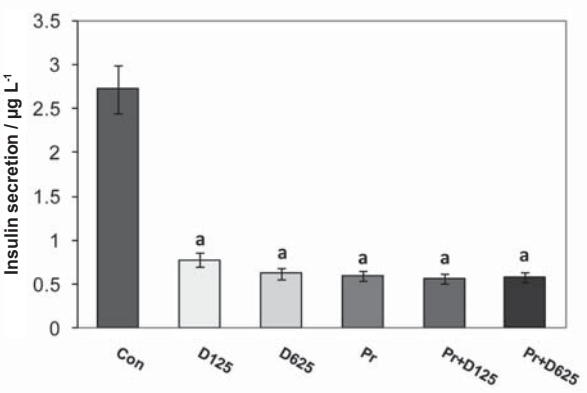

Figure 6 *Insulin release from islets of Langerhans incubated for 30 min in the presence 8 mmol L^{-1} glucose as control (Con) plus diazinon alone or in combination with propranolol (Pr). Diazinon alone at doses of 125 µg mL^{-1} and 625 µg mL^{-1}, 0.1 µmol L^{-1} propranolol alone, or diazinon in combination with 0.1 µmol L^{-1} propranolol were used. Values are means±SEM of 3 experiments. [a] $P<0.05$ for difference from control.*

atropine potentiated diazinon-induced hyposecretion of insulin. Antioxidant alpha-tocopherol showed the same protection level like ACh. Our results confirm that diazinon acts both through the cholinergic system and free radical-induced stress. The controlling role of the cholinergic system in insulin release has been proposed by studies in mice (20), rats (21), monkeys (22), and humans (23). ACh is believed to be a potent secretagogue of both insulin and glucagon while the autonomic nervous system affects glucose-dependent insulin secretion (24, 25). There is evidence that carbachol, a cholinergic agonist, increases glucose-induced insulin secretion from isolated rat islets (26), which corresponds to our results showing that ACh can recover diazinon-induced drop in insulin secretion. In all our experiments, insulin secretion was stimulated by 8 mmol L^{-1} glucose to better track changes caused by interactive compounds. In these conditions, addition of stimulants like ACh does not increase insulin secretion over control, because it has already reached the highest possible secretory level. In beta-cells, ACh is thought to bind to muscarinic M3 receptor subtype and to exert complex effects eventually leading to increased insulin secretion. Activation of phospholipase C (PLC) generates diacylglycerol (DAG) that activates protein kinase C (PKC), thereby increasing the efficiency of free cytosolic calcium concentration on exocytosis of insulin granules. ACh activates intracellular movement of secretory granules as a result of muscarinic mobilization of intracellular calcium (27). Inositol-3-phosphate (IP3) produced by PLC causes a rapid elevation of calcium by mobilising calcium from the endoplasmic reticulum. The resulting fall in calcium in the organelle produces a small capacitative calcium entry (25). Atropine, an agent that blocks muscarinic receptors, decreased glucose-induced insulin release. It has been reported that the inhibitory effect of clozapine (which strongly binds to muscarinic M3 receptor) or atropine is not observed under non-stimulatory glucose concentration, but is evident at 7.0 mmol L^{-1} glucose (28). Confirming the role of ACh, our data indicate that atropine reduced 8 mmol L^{-1} glucose-stimulated insulin secretion when used alone, but, interestingly, it potentiated the diazinon-induced effect (Figure 2). In addition, the inhibitory effect of diazinon is partly diminished by ACh. This confirms the controversy between the proposed mechanisms of OP-induced changes in hormonal glucose control by pancreas and also non-hormonal changes by organs such as liver or muscles (11, 24, 30-38). The cholinergic system seems to be responding well to ACh and atropine, which suggests that the method used is reliable, but diazinon most probably works by a mechanism other than cholinergic. In fact, in this *in vitro* model, this kind of interaction between diazinon and the cholinergic system is not too surprising because the amount of cholinesterase in the islets seems to be insufficient, and therefore the effects observed here are possibly mediated only by diazinon and not by ACh.

Our data also showed that even though phenoxybenzamine, propranolol, and nifedipine, inhibited glucose-induced insulin release, they could not change diazinon-induced insulin reduction. The reduction of glucose-stimulated insulin confirms the efficiency of the selected blockers and, again, the reliability and reproducibility of this *in vitro* model. It turns out that the adrenergic system and calcium channels do not have the main role in diazinon-induced changes.

Our experiment with alpha-tocopherol sheds more light on the effects of diazinon in the presence of ACh and atropine. OPs are known to produce oxidative stress by generating free radicals and modifying the antioxidant defence system. Many studies have already indicated that enzymes associated with antioxidant defence mechanisms are altered under the influence of OPs, and that lipid peroxidation is one of the molecular mechanisms involved in OP-induced cytotoxicity (8, 11, 13, 29-31, 35-38). Further supportive evidence comes from studies indicating diazinon-induced free radical damage to pancreatic B-cells as the cause of hyperglycaemia in animals (17, 30). Our study has shown that alpha-tocopherol is a potent antioxidant that can restore diazinon-reduced insulin secretion, most probably by preventing free radical toxic damage. This is supported by a report on increased glucose-stimulated insulin release one day after exposure of islets to alpha-tocopherol (39). In addition, pre-incubation of pancreatic islet cells with alpha-tocopherol significantly improved their resistance to toxic doses of nitric oxide (40). A recent study (41) has shown that when diazinon was administered *in vivo* at doses of (15 to 60) mg kg^{-1} to rats, plasma insulin decreased while C-peptide concentrations increased. In addition, diazinon increased the activity of glutamate dehydrogenase (GDH), decreasing at the same time the expression of GDH gene. This suggests that GDH participates in diazinon-induced changes in the release of immature insulin. Therefore, it is reasonable to conclude that diazinon induces secretion of immature insulin from isolated islets.

Our results and the above-mentioned evidence suggest that diazinon can affect insulin secretion mainly by disturbing the balance between free radicals and antioxidants in the islets of Langerhans and by inducing toxic stress and release of immature insulin. A number of evidence on the relationship between oxidative stress and diabetes (32-34, 42-47) even by OP compounds (48) supports this conclusion, and calls for further research of the beneficial effects of antioxidants (49). Our future work will focus on the balance between insulin secretion, antioxidant levels, and islet cytotoxicity.

Acknowledgement

This study was granted by the Tehran University of Medical Sciences.

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Sažetak

USPOREDBA DJELOVANJA BLOKATORA KALCIJEVIH KANALA, BLOKATORA AUTONOMNOGA ŽIVČANOG SUSTAVA TE INHIBITORA SLOBODNIH RADIKALA NA HIPOSEKRECIJU INZULINA IZ IZOLIRANIH LANGERHANSOVIH OTOČIĆA ŠTAKORA UZROKOVANU DIAZINONOM

U osoba izloženih organofosfatnim insekticidima zamijećen je nastanak hiperglikemije. Svrha je ovog istraživanja bila usporediti djelovanje blokatora kalcijevih kanala, alfa i beta-adrenergičkih i muskarinskih receptora te inhibicije slobodnih radikala na lučenje inzulina iz Langerhansovih otočića izoliranih iz štakora tretiranih diazinonom.

Otočići su izolirani iz gušterače štakora s pomoću standardnog postupka digestije kolagenazom, odvajanja centrifugiranjem i metodom ručnog probira (engl. *hand-picking*) te su kultivirani u inkubatoru pri 37 °C i 5 % CO_2. Pokusne su kulture inkubirane s 1 mL glukoze u koncentraciji od 8 mmol L^{-1} te diazinonom u dozi od 125 μg mL^{-1}, odnosno 625 μg mL^{-1}. U kontrolu je dodana samo glukoza u koncentraciji od 8 mmol L^{-1}. Nakon toga je u kulture dodan jedan od sljedećih agenasa: 30 μmol L^{-1} atropin, 100 μmol L^{-1} ACh + 10 μmol L^{-1} neostigmin, 0,1 μmol L^{-1} propranolol, 2 μmol L^{-1} nifedipin, 50 μmol L^{-1} fenoksibenzamin, odnosno 10 μmol L^{-1} alfa-tokoferol. U svim je pokusima diazinon značajno smanjio lučenje inzulina, s time da je doza od 125 μg mL^{-1} dovela do 62,2%-tne inhibicije, a doza od 625 μg mL^{-1} do 64,38%-tne inhibicije lučenja inzulina, što upućuje na djelovanje neovisno o dozi. Acetilkolin i alfa-tokoferol su ponovno potaknuli lučenje inzulina, za razliku od atropina koji ga je dodatno smanjio. Primjena blokatora alfa i beta-adrenergičkih receptora te blokatora kalcijevih kanala nije utjecala na djelovanje diazinona.

Autori zaključuju da diazinon utječe na lučenje inzulina ponajviše narušavanjem ravnoteže između slobodnih radikala i antioksidansa u Langerhansovim otočićima te dovodi do toksičnoga stresa.

KLJUČNE RIJEČI: *gušterača, oksidativni stres, organofosfati, štakori*

CORRESPONDING AUTHOR:

Professor Mohammad Abdollahi
Laboratory of Toxicology, Faculty of Pharmacy and
Pharmaceutical Sciences Research Center (PSRC)
Tehran University of Medical Sciences (TUMS)
Tehran 1417614411, Iran
E-mail: *mohammad.abdollahi@utoronto.ca*