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Positive Response of Isolated Rat Pancreatic Islets to IMOD; Hopes for Better Transplant Outcome and Graft Function

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ABSTRACT

IMOD as a multi-herbal-selenium electromagnetically-treated combination was tested for its potential on the function and level of Reactive Oxygen Species (ROS) in isolated rat pancreatic islets. After Laparotomy, pancreas were removed and islets were isolated and incubated in RPMI 1640 for 24 h and then islets were separated and divided into several tubes containing ten in each. IMOD at logarithmic doses (0.1, 1, 10, 100 and 1000 ppm) were added to islets and incubated for 24 h and then static insulin secretion, at low dose (2.8 mM) glucose and high dose (16.7 mM) glucose was tested. Cells viability and ROS were also determined. IMOD at 0.1 ppm increased islet insuhn secretion in response to glucose in both basic and stimulation levels. This drug at 1 ppm dose only increased insulin concentration in stimulation levels of glucose. IMOD at 10, 100 and 1000 ppm decreased insulin secretion in both levels of glucose. The viability at the doses of 0.1 and 1 ppm increased. The result of ROS test showed a decrease although significant, at the lower dose (0.1 μM) but increased dose-dependently at the doses of 10, 100 and 1000 ppm. IMOD has significant anti oxidative effects at low doses and improves viability and insulin secretion of isolated islets in both basic and stimulation levels of glucose. Pretreatment with IMOD may improve transplant outcome and graft function. Its effect in insulin release maybe promising in treatment of diabetes.

Key words: Oxidative stress, IMOD, isolated rat pancreatic islets, toxicology, pharmacology

INTRODUCTION

Diabetes Mellitus is a global disease with high economic and social burden. Islet transplantation can today be offered to selected patients with type-1 diabetes who are non-responsive to drugs. Despite many advances in protocols for pancreatic islet transplantation, yet it cannot compensate complete body insulin requirement and thus pancreatic islets derived from multiple donors are needed. It is believed that in the transplant setting, islets of Langerhans are faced with various types of stress related to the isolation and transplantation procedure which trigger a cascade of cell signaling pathways that compromise their function and viability. For instance, apoptosis during the initial stages of islet transplantation prior to stable engraftment results in non-functionality. Newly transplanted islets are essentially avascular and thus islets must pass the initial period of

hypoxic ischemia until the process of revascularization is completed. This ischemic microenvironment produces oxidative stress condition that is detrimental to transplanted islets (Mohseni-Salehi-Monfared *et al*., 2009).

Islets are essentially vulnerable to oxidative-induced damage because they have inherently decreased antioxidant capacity (Rahimi *et al*., 2005). The antigen-independent complexities of islet transplantation increase the incidence of primary graft non-functionality and β-cell death, thus requiring protection for islets at early stages of the transplant procedure (Irani *et al*., 2009). Prevention of oxidative stress by means of more powerful compounds may further improve the survival of isolated islets in culture and *in vivo* (Mohseni-Salehi-Monfared *et al*., 2009; Hasani-Ranjbar *et al*., 2008).

Setarud (IMOD) is the outcome of an invention referring to a method for preparing an herbal extract from a mixture of *Rosa canina*, *Tanacetumvulgare* and *Urtica dioica* comprising selenium and urea treated by pulsed electromagnetic field of high frequency that has been patented in USA and Europe (Novitsky *et al*., 2007) for its efficacy in Human Immunodeficiency Virus (HIV) infection by increasing CD4 and reduction of tumor necrosis factor alpha (TNF-α). The herbs used in this complex have strong anti free-radical potentials and useful in oxidant-related diseases (Hasani-Ranjbar *et al*., 2009). The benefit of IMOD in autoimmune experimental diabetes (Mohseni-Salehi-Monfared *et al*., 2010) and in human severe sepsis (Mahmoodpoor *et al*., 2010) have been recently reported. Commonly used anti-rejection drugs are excellent at inhibiting the adaptive immune response; however, most are harmful to islets and do not protect well from oxidative damage resulting from islet isolation and ischemia-reperfusion injury. The aim of this investigation was to examine probable benefit of IMOD on *in vitro* islet viability and function.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from Sigma-AldrichChemie (Gmbh, Munich, Germany) unless otherwise stated. IMOD™ was obtained from Parsrus Research Group (Tehran, Iran). Rat specific insulin ELISA kit was purchased from (Mercodia, Sweden).

Experimental animals: Male adult Wistar rats (>12 weeks old), with a weight of approximately 250±25 g, were used as pancreatic islets donors. All of the animals were treated according to the National Health Ethical Guidelines for experimental animal studies.

Pancreatic islet isolation and culture: After accommodation of rats to lab environment, rats were anesthetized with intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1}) and underwent laparotomy; the common bile duct was ligate at the duodenal end and cannulated at its the duodenal side. Then, the rat pancreas was distended by injecting 10 mL of Krebs buffer ([in grams per liter] 8 NACL, 0.27 KCL, 0.42 $NaHCO_3$, 0.06 NaH_2PO_4, 0.05 $MgCL_2$, 2.38 HEPES, 0.22 $CaCL_2.2H_2O$, 0.5 glucose. $1H_2O$, pH = 7.4) into the duct. Distended pancreas was excised carefully from duodenum and preserved in a plate within Krebs buffer solution. The pancreas was cut into 1-2 mm pieces to increase the surface area and providing conditions for digestive enzyme collagenase to break down the tissue surrounding the islets. After separation of fat tissue, islets were washed by Krebs-HEPES buffer three times. Then the extracted tissue was placed in falconin Krebs solution and centrifuged with 1700 rpm 60 sec for two times and

0.5% BSA was added for completion of digestion. Islets were thereafter isolated by hand picking with sampler under a stereomicroscope and handpick islet was incubated in culture media which contain RPMI-1640 medium, in 5% CO_2 at 37°C for 15 h.

IMOD *in vitro* dose optimization: Optimization of dose was done by pretreating islets with various concentrations (0.1, 1, 10, 100, 1000 μl L^{-1} or ppm) of IMOD for 24 h. IMOD solution is dispersed as vials containing 125 mg of active ingredients in 4 mL solution. The effective *in vivo* dose of IMOD is 20 mg kg^{-1} in rat (Baghaei *et al*., 2010).

Insulin secretion: Isolated islet divided in several groups. Each group contained ten islets in 1 mL Krebs medium alone or in combination with different concentrations of IMOD. After 24 h incubation, medium which contain different concentrations of IMOD was removed and islets were washed twice by Krebs-HEPES buffer and preincubated for 30 min at 37°C at a level of 2.8 mM glucose. Then, all groups were treated in two glucose concentrations of 2.8 and 16.7 mM as basal and stimulant doses, respectively and subsequently incubated for 30 min at 37°C. The supernatants were collected and stored in separate microtubes. Insulin assays were performed with rat insulin ELISA kit (Mercodia, Sweden) according to the manufacturer's protocol.

Metabolic activity of islets by MTT: 3-4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), a yellow tetrazole is reduced to purple formazan in living cells. After 24 h incubation with different concentrations of IMOD, medium was removed and islets were washed twice by Krebs-HEPES buffer. Then 20 μL from MTT solution was added to islets and incubated for 4 h. At the end, 100 μL DMSO solution was added to culture medium and after 30 min, the absorbance was determined at 570 nm by ELISA reader.

Reactive Oxygen Species (ROS) assay: The intracellular formation of reactive oxygen species was measured using 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA). The non-fluorescent compound DCFH-DA penetrates into the cell and is cleaved by intracellular esterase, resulting in the formation of 2',7'-dichlorodihydrofluorescin (DCFH), the oxidation of which (due to oxidative stress) generates the fluorescent compound dichlorofluorescein. Ninty six wells plate were used to measure ROS. After homogenization of each ten islet isolated from pancreatic tissue by extraction buffer and centrifuging the homogenized solution (with 5000 rpm/5 min), the extracted solution was separated and collected in separate micro-tube. To measure ROS, 162 μL of the buffer assay with 10 μL DCFH-acetate with in each of the plates sink down into each well of plates. Then, 50 μL of homogenized samples was added to it. After incubation the samples for 15 min at 37°C for 60 min to absorb the changes (excitation: 488 nm/emission: 528 nm), changes in absorbance were measured by ELISA fluorimeter. All values were standardized by the amount of total protein in each well (Momtaz *et al*., 2010).

Protein assay: To measure total protein concentration of islets, diluted samples were mixed with Bradford reagent dye and after 5 min, the absorbance was measured at 595 nm by the spectrophotometer. Albumin was used as standard.

Statistical analysis: Data were analyzed by StatsDirect statistical software version 2.7.8. Results are expressed as the Mean±SEM of from three separate tests examined duplicates. One-way analysis of variance followed by Tukey'sposthoc multiple comparisons were used as the statistical tests. The $p<0.05$ was considered statistically significant.

RESULTS

Effect of IMOD on islet's insulin release: As seen in Fig. 1, administration of IMOD in 0.1 ppm increased insulin secretion in basal glucose (2.8 Mm) and stimulated (16.7 mM) concentrations. However, administration of IMOD (1 ppm) had no significant effect on insulin secretion from islet in basal state but significantly increased insulin secretion from islets at high glucose concentration (16.7 mM) when compared with the matched control. IMOD at doses of 10, 100 and 1000 ppm significantly decreased secretion of insulin in both basal and stimulated glucose states.

Effect of IMOD on islet's metabolic activity (MTT assay): Results are shown in Fig. 2, as percent change in relation to control. IMOD significantly increased viability in 0.1, 1, 10, 100 and 1000 ppm, among them the maximum change was observed in 1 ppm. Despite increase in viability of islets in lower doses of IMOD, viability was progressively decreased in concentrations of 10,100 and 1000 ppm significantly different from that of 1 ppm.

Effect of on islet's ROS: Results are shown as percent change in relation to control (Fig. 3). IMOD significantly increased ROS in 100 and 1000 ppm. However, there was no significant change in ROS at doses of 1 and 10 ppm but a significant reduction at dose of 0.1 was observed. Despite the lack of significant differences in ROS levels at doses of 1 and 10 ppm, the observed changes in the concentrations studied were dose-dependent.

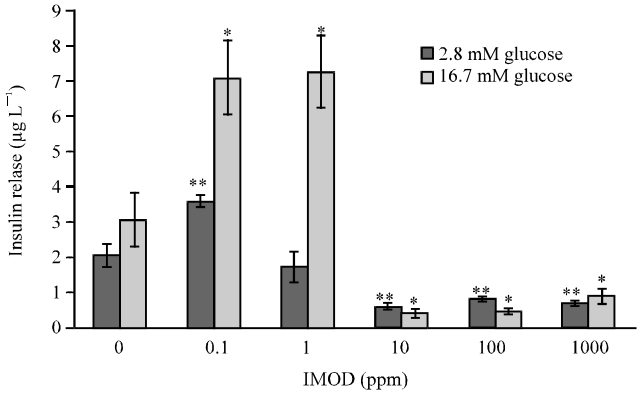

Fig. 1: Effects of IMOD on release of insulin from isolated rat islets. After 24 h incubation in the exposure of various concentrations of IMOD, islets were incubated for 30 min in the presence of various concentrations of glucose as basal (2.8 mM) or stimulant (16.7 mM). Data are expressed as Mean±SEM of 3 different experiments (each experiment was performed in duplicate). *Significantly different from control group ($p<0.05$); **Significantly different from control group ($p<0.01$)

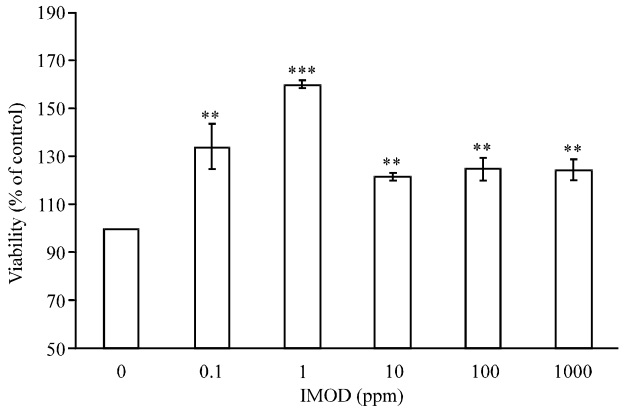

Fig. 2: Effects of IMOD on viability of isolated rat islets. Data are expressed as Mean±SEM of 3 different experiments (each experiment was performed in duplicate). **Significantly different from control group ($p<0.01$); ***Significantly different from control group ($p<0.001$)

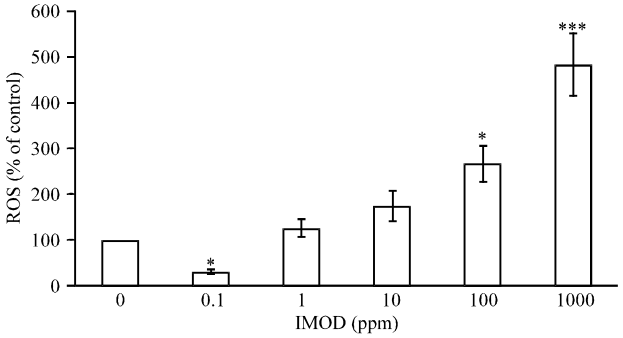

Fig. 3: Effects of IMOD on production of ROS in isolated rat islets. Data are expressed as Mean±SEM of 3 different experiments (each experiment was performed in duplicate). *Significantly different from control group ($p<0.05$); ***Significantly different from control group ($p<0.001$)

DISCUSSION

The present results show that IMOD in dose of 0.1 and 1 ppm enhances the function of islets by increasing insulin secretion in the stimulated state; however only in dose of 0.1 ppm it increased insulin secretion in basal state. In this study, we showed that IMOD increases viability of cells in all doses used in comparison to controls where the maximum effect was observed by dose of 1 ppm. Although, the current study confirms that IMOD in doses of 100 and 1000 ppm increases oxidative cell damage in pancreatic beta cells but, in the dose of 0.1 ppm its antioxidative effects was evident.

Enhancement of insuhn secretion at high glucose level (16.7 mM) supports that IMOD does not disturb islet cells membrane but provokes glucose-induced insulin secretion in beta cells. Insulin secretion in stimulated condition may indicate cell membrane stability and indicate functional viability. The unexpected effects of IMOD at higher doses were seen in MTT assay which gives the impression on sensitivity of islets to IMOD and the fact that proper doses of IMOD must be used *in vitro*. IMOD increases viability in all concentration despite a decrease in insulin release in doses

above 1 ppm (10 to 1000 ppm). These findings suggest that effect of IMOD on secretion of insulin is independent of its effect on islet viability and may be due to its secretory stimulation or other unmeasured factors. Another possible reason for the discrepancy is that insulin content may have been overestimated due to non-specific intracellular insulin release into the culture medium from damaged islet cells during incubation especially in basal state.

In comparison of effects of IMOD on insulin secretion and viability, IMOD increased insulin secretion only at 0.1 and 1 ppm but increased viability in all doses. This means that viability is a complex factor independent of insulin secretion.

When insulin secretion and ROS production are compared, it is seen that by increasing of ROS in doses higher than 1 ppm results in lower release of insulin. This suggests that ROS causes islet cells damage and reduces their functional viability.

The current study confirmed that IMOD in doses of 100 and 1000 ppm induces oxidative cell damage in islets most probably through pro-oxidant activities of islets. We have demonstrated that IMOD in doses of 100 and 1000 ppm functions as a pro-oxidant to activate pancreatic beta cell damage but despite an increase in ROS, viability of cell increases.

Also, based on observed antioxidative effects in dose of 0.1 ppm, low dose of IMOD may increase islet's antioxidative enzymes such as catalase, superoxide dismutase and glutathione peroxidase in favor of defense against oxidative stress. Previous studies have demonstrated antioxidative activity of IMOD (Mohseni-Salehi-Monfared *et al*., 2009; Baghaei *et al*., 2010; Agha-Hosseini *et al*., 2011), or its main component Urtica dioica (Mehri *et al*., 2011). In experimental colitis, IMOD remarkably reduced histological scores of colitis through controlling of TNF-α, interleukin-1β, myeloperoxidase and lipid peroxidation (Baghaei *et al*., 2010). In addition, in experimental immune model of diabetes, IMOD decreased pancreatic lipid peroxidation, myeloperoxidase, TNF-α and interleukin-1β (Mohseni-Salehi-Monfared *et al*., 2009). Therefore, *in vivo* data do not support dual function of IMOD as an anti-oxidant or pro-oxidant while as shown in the present *in vitro* study, nanomolar concentrations of IMOD may exert beneficial antioxidant activity whereas pro-oxidant activity may be generated at micromolar concentrations. However, we found evidence of antioxidative activity at nontoxic concentrations.

Therefore, oxidative or antioxidative effects of IMOD *in vitro* depend on doses that are used. Of course, this effect of IMOD should be tested in other cell lines *in vitro* to examine if it is cell-dependent or not. Taken together, the present study indicates that IMOD in doses of 100 and 1000 ppm mediates formation of highly toxic radicals which in turn induces oxidative cell damage in the rat islets. A clear understanding of the pro-oxidant mechanisms of IMOD or antioxidant at physiological levels needs to be established by measuring apoptotic and necrotic pathways (NF-κB-DNA binding) and cytokines. Finally, measurements of islets viability, insulin secretion as well as ROS conclusively demonstrate that pretreatment with IMOD at dose of 0.1 ppm rescues islets from death or dysfunction via scavenging ROS. Pretreatment with IMOD may improve transplant outcome and graft function but must be tested in a good model. Its effect in insulin release maybe promising in treatment of diabetes.

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