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On the Benefit of Pure Glycyrrhizic Acid on the Function and Metabolic Activity of Isolated Pancreatic Langerhans Islets *in vitro*

^{1,3}Mahban Rahimifard, ^{2,3}Mona Navaii-Nigjeh, ³Amir Nilli-Ahmadabadi, ³Nazila Pourkhalili, ³Maryam Baeeri, ³Azadeh Mohammadirad and ³Mohammad Abdollahi

¹Faculty of Science and Agriculture, Payame Noor University of Tehran, Tehran, Iran

²Faculty of New Sciences and Technologies, University of Tehran, Tehran, Iran

³Faculty of Pharmacy and Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran 1417614411, Iran

Corresponding Author: Mohammad Abdollahi, Faculty of Pharmacy and Pharmaceutical Sciences Research Centre, Tehran University of Medical Sciences, Tehran 1417614411, Iran

ABSTRACT

Glycyrrhizic Acid (GA) a major component of licorice, has been reported to have potent antioxidant effects and used widely throughout the world. In the present study, the effects of GA on the function, viability and level of Reactive Oxygen Species (ROS) in isolated rat pancreatic islets were evaluated. After Laparotomy, pancreas was removed and islets were isolated and incubated in RPMI 1640 for 24 h and then islets were separated. GA at logarithmic doses (1, 10, 100 and 1000 μ M) were added to islets and incubated for 24 h and then static insulin secretion was tested. Also, viability of cells and their ROS level were determined using Mitochondrial Toxicity Test (MTT) and fluorometric assay. Then islets were stained by dithizone and observed under microscope. The results of MTT test indicated that rang of 1-100 μ M of GA is safe. In the dose of 1000 μ M, GA increased ROS and reduced viability of islets. GA at 1 μ M significantly increased secretion of insulin via isolated islets in the presence of stimulation level of glucose (16.7 mM). Results of dithizone staining showed a reduction in live cells at high dose of GA. The LC_{50} study was done to determine the toxicity of GA on rat pancreatic islets and a 24 h LC_{50} of 15 mM was found. GA showed remarkable anti oxidative effects at low doses and improved islet's viability and insulin secretion in stimulation level of glucose. Interestingly, high dose of GA induced oxidative stress and reduced function of islets. The results of the present study indicate that GA is a good candidate to be examined in islet transplantation procedures to maintain islets viable and functional.

Key words: Oxidative stress, glycyrrhizic acid, isolated rat pancreatic islets

INTRODUCTION

Many of people in the world are diabetic and most of them use traditional medicine for their health needs. Among herbal medicines, *Glycyrrhiza glabra* (Licorice) is an encouraging medical plant reported to have potent antidiabetic effects and has been used widely in various regions of Iran for many years (Irani *et al.*, 2010). Today root of *Glycyrrhiza glabra* is known to have anti-oxidative properties, anti-inflammatory and anti-viral effects. It has also important role in treatment of fever, diarrhea and rheumatism (Vispute and Khopade, 2011). Glycyrrhizic acid (GA) is the main active component of *Glycyrrhiza glabra*'s root that is responsible for most of positive biological effects (Eu *et al.*, 2010; Asl and Hosseinzadeh, 2008; Dehpour *et al.*, 1999).

Oxidative stress was defined several years ago as the imbalance between pro-oxidative and anti-oxidative molecules in a biological system and happens when body oxidants overwhelm body defensive molecules (Abdollahi *et al.*, 2004). Increased oxidative stress is a significant contributor to development and progression of Diabetes Mellitus (DM) and its complications such as β -cell failure (Rahimi *et al.*, 2005; Monfared *et al.*, 2009). Because β -cells have the lowest cell antioxidant capacity, they are essentially vulnerable to oxidative-induced damage rather than other cells (Larijani *et al.*, 2011). There are numerous evidences about role of the antioxidants in improvement of function of β -cells that could be addressed in importance of oxidative stress in pathogenesis of β -cell failure (Pourkhalili *et al.*, 2012).

One of the most important goals in islet transplantation is to improve islet's maintenance and functionality through inhibition of oxidative stress process. Regarding antioxidant potential of GA, we were interested to test it on viability and functionality of isolated rat pancreatic Langerhans islets.

MATERIALS AND METHODS

Chemicals: All chemicals used in this study were obtained from Sigma-Aldrich Co. (GmbH, Munich, Germany). Rat specific insulin ELISA kit was obtained from Mercodia (Sweden) and glycyrrhizic acid ammonium salt hydrate was obtained from Carl Roth Company.

All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Isolation and incubation of islets: The islets were isolated from male adult Wistar rats (2-3 months), with a weight of $\sim 250 \pm 25$ g. All animal care were performed according to the animal welfare Act approved by Pharmaceutical Sciences Research Center Ethics Committee.

After the rats were adapted to lab environment, they were anesthetized by injection of sodium pentobarbital (60 mg kg^{-1}). Pancreas was inflated by injecting 10 mL of Krebs buffer (8 g L^{-1} : NaCl, 0.27 g L^{-1} : KCl, 0.42 g L^{-1} : NaH_2PO_4 , 0.05 g L^{-1} : MgCl_2 , 2.38 g L^{-1} : HEPES, 0.22 g L^{-1} : $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g L^{-1} : glucose. $1\text{H}_2\text{O}$ at $\text{pH} = 7.4$) into duodenal duct. Inflated pancreas was removed carefully and cleaned from lymph nodes, fat and blood vessels. It was kept in Krebs buffer on ice, cut into small pieces and washed two times by centrifuging at 3000 g for 60 sec. Then collagenase enzyme was added to pancreas at 37°C for 10 min. This time, pancreas was washed two times by Krebs buffer with 0.5% Bovine Serum Albumin (BSA) and again centrifuged twice at 3000 g for 60 sec. At that time, tissue surrounding islets and fat tissue were removed and the purified islets were stored in the falcons. Under stereomicroscope, islets of the similar size were isolated by hand picking using a sampler. Then they were cultured for 16 h at 37°C with 0.5% CO_2 in RPMI 1640 medium containing fetal bovine serum, antibiotic and 8.3 mmol L^{-1} glucose (Pourkhalili *et al.*, 2012).

Doses of GA: Various concentrations (1, 10, 100, $1000 \mu\text{L L}^{-1}$) were made in RPMI medium culture and exposed to the islets for 24 h at 37°C .

Cell viability assay: Cell viability of GA was measured by dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide named Mitochondrial Toxicity Test (MTT). After 24 h incubation with various doses of GA, the medium was removed and washed two times by Krebs-HEPES. Then $20 \mu\text{L}$ of MTT (0.5 mg mL^{-1}) was added and incubated for 3 h at 37°C . The violet crystal was dissolved by Dimethyl Sulfoxide (DMSO) and after 30 min, the optical density was measured at

570 nm using ELISA reader. For this test, in addition to doses of 1-1000 μM , the 10000 μM was also tested in order to establish an LC_{50} value (concentration to inhibit 50% of cells) of GA on pancreatic islet cells.

Diphenylthiocarbazone staining: Dithizone (10 mg) was completely dissolved in 10 mL of DMSO and was stored at -20°C . The working solution (pH 7.8) was prepared immediately prior to use by diluting the stock solution 1:10 in Phosphate Buffered Saline (PBS). For each dish, 2 mL of the dithizone solution was added and incubated for 30 min at 37°C .

Insulin secretion: Function of islets was assessed by insulin secretion in glucose static incubation. After exposing groups of islets by different concentration of GA in 24 h, the media and GA were removed from islets by twice washing with Krebs-HEPES buffer. Then they were incubated by 2.8 mM glucose for 30 min. After that, islets were divided into two groups, the first were treated by 2.8 mM glucose (the basal dose) and the second by 16.7 mM glucose (stimulant dose) for 30 min at 37°C . Islets were centrifuged and Insulin assay in the supernatant were performed using rat insulin ELISA kit.

Intracellular ROS: Intracellular Reactive Oxygen Species (ROS) was measured using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Islets were collected and washed three times and homogenized by extraction buffer, then they were centrifuged and 10 μM 2',7'-dichlorodihydrofluorescein (DCFH) was added to 162 μM supernatant. 2',7'-dichlorofluorescein-based (DCF-based) detection of unspecified ROS was performed by loading islets with the nonfluorescent, reduced form of DCF, 2',7'-dihydrochlorofluorescein-di-acetate (H_2DCF) which is oxidized to DCF-di-acetate in the presence of ROS. After 30 min incubation in 37°C , the ELISA fluorimeter was used to measure absorbance every 10 min up to 60 min as set up in our lab previously (Momtaz *et al.*, 2010).

Protein assay: Total protein content was determined by adding Bradford reagent dye to diluted samples and using albumin as standard. Absorbance was read at 595 nm by ELISA reader and protein concentration was calculated from the calibration curve.

Statistical analysis: Data were expressed as Mean \pm SEM and compared using one way analysis of variance (ANOVA). The $p < 0.05$ was considered statistically significant.

RESULTS

The effect of GA on viability of islets: As MTT is shown in Fig. 1, the metabolic activity of islets in 1, 10 and 100 μM of GA did not significantly change but at 1000 and 10000 μM , the viability decreased. These data pointed out an LC_{50} of 15000 μM for GA.

Diphenylthiocarbazone staining of isolation islets: In concentration of 1 and 10 μM of GA, the number of live red islets and staining intensity were almost the same as controls (Fig. 2a-c) but lessening in the number of stained cells was realized in higher doses such as 100 and 1000 μM of GA (Fig. 2d-e). It means that higher doses of GA cause death of islets as shown in Fig. 3.

The effect of GA on rate of islet's insulin release: As seen in Fig. 4, concentration of insulin in 1 μM GA showed a little increase in basal phase but it significantly increased in stimulated phase

(16.7 mM). Changes in 10 and 100 μ M GA in both basal and stimulated phases did not significantly change the release of insulin. Comparing the dose of 1000 μ M with controls showed a significant diminution in the presence of high concentration of glucose (16.7 mM).

The effect of GA on islet's ROS: Results in Fig. 5, show that ROS in concentrations of 1, 10 and 100 μ M of GA is decreased. The maximum change of ROS was observed in dose of 1 μ M. The dose of 1000 μ M significantly increased ROS.

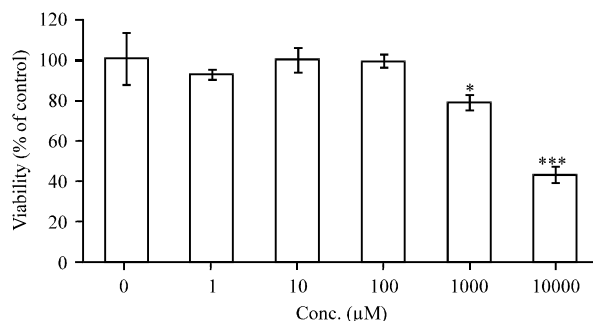


Fig. 1: Effects of different concentrations of glycyrrhizic acid on viability of isolated rat pancreatic islets. Data are expressed as Mean \pm SEM of 3 different experiments (each experiment was performed in duplicate), ****Significantly decrease from control group at $p < 0.05$ and $p < 0.001$, respectively

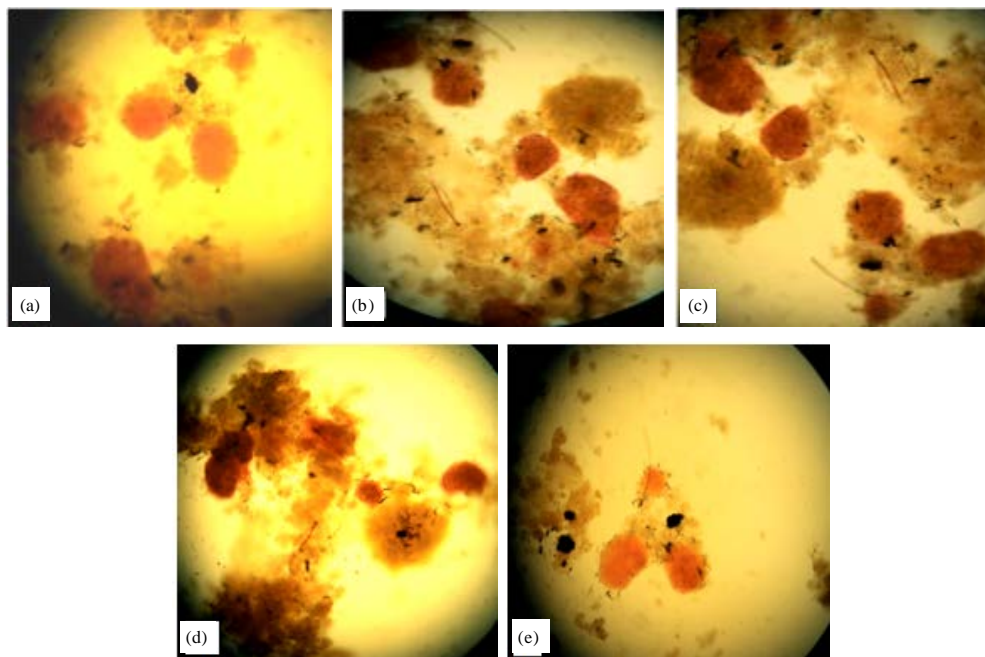


Fig. 2(a-e): Dithizone staining of isolated rat pancreatic islets treated with, (a) Control group, (b) 1 μ M: 50% cells are red, (c) 10 μ M: 50% cells are red, (d) 100 μ M: 33% cells are red and (e) 1000 μ M: 25% cells are red; of glycyrrhizic acid (GA), Live cells are red and dead cells are light brown, Magnification x40

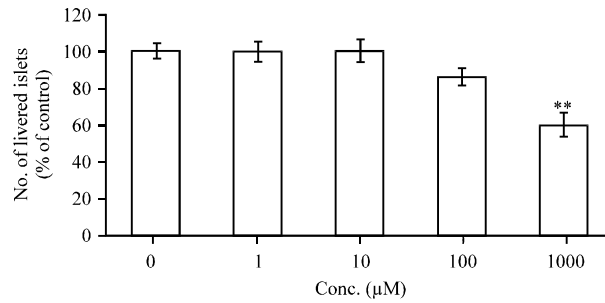


Fig. 3: Effects of different concentrations of glycyrrhizic acid on number of isolated rat pancreatic live islets, stained with dithizone and were become red, There were 12 islets in each group, Data are expressed as Mean±SEM of 3 different experiments, each experiment was performed in duplicate, **Significant increase from control group at $p<0.01$

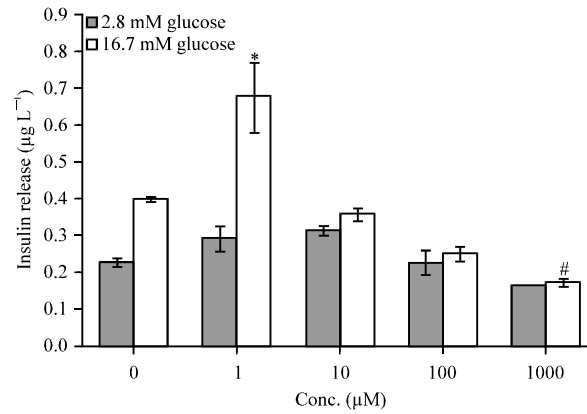


Fig. 4: Effects of different concentrations of glycyrrhizic acid on releasing insulin from isolated rat pancreatic islets, Data are expressed as Mean±SEM of 3 different experiments, each experiment was performed in duplicate, *#Significant increase and decrease, respectively, when compared with control group at $p<0.05$

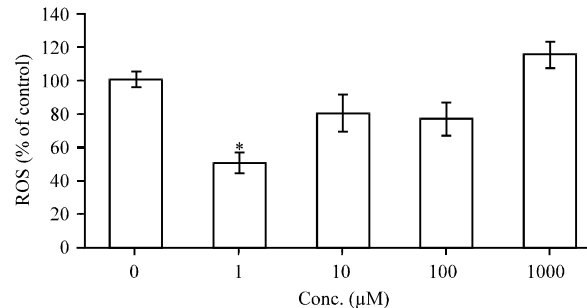


Fig. 5: Effect of different concentrations of glycyrrhizic acid on level of reactive oxygen species (ROS) in isolated rat pancreatic islets, Data are expressed as Mean±SEM of 3 different experiments, Each experiment was performed in duplicate, *Significant decrease when compared to control group at $p<0.05$

DISCUSSION

Licorice is a very popular herb for treating diabetes and glycyrrhizic acid has been shown its primary bioactive compound (Kao *et al.*, 2009). The anti oxidative effects of this component are investigated on different cells in various aspects.

In the present study, we observed a significant decrease in rate of ROS and an increase in secretion of insulin by dose of 1 ppm of GA. GA has been previously found to reduce ROS in other cells like neuronal cells by elevating the intracellular antioxidant system (Kao *et al.*, 2009). Present results show that this active compound provokes glucose-induced insulin secretion in islets and increases the reaction to stimulated dose of glucose by stabilizing cell membrane, islet's function and viability. Reduction of ROS and increase in simultaneous secretion of insulin at low dose support that insulin gene expression is positively regulated to better functioning of beta cells. Naturally, excessive concentrations of ROS would reduce insulin's gene expression and secretion and even would damage the islets as was observed by use of GA at higher concentrations. By doing MTT, the LC₅₀ value of GA the major bioactive triterpene glycoside of licorice root extracts was found 15000 μ M. Although the mechanism of antioxidant property of GA has not been fully elucidated, its glutathione peroxidase-like activity seems to play an important role in its anti oxidative effects (Chan *et al.*, 2003).

Measuring viability of the islets demonstrated that effective dose (1 ppm) of GA in ROS and insulin test is safe and it doesn't decrease the viability as confirmed by present diphenylthiocarbazone staining of islets (Fig. 2). The best finding of the present study is that GA at 1 ppm is a nontoxic effective dose. The results of the present study indicate that GA is a good candidate to be further examined in islet transplantation to maintain functionality and viability of islets. Regarding these capacities of GA, it is likely to see beneficial effects in diabetes as already confirmed for *Satureja* species with the same potentials (Momtaz and Abdollahi, 2010). Further studies should be focused to combine GA with other antioxidants with similar effects (Hosseini and Abdollahi, 2012; Mohammadi *et al.*, 2011) to reach a strong mixture useful to maintain isolated islets viable and functional.

CONCLUSION

On the basis of existing documents, the suggestion of authors is to test combination of GA with essence of *Satureja* or IMOD with the hope to reach better viability and functionality of isolated islets.

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