Inhibitory action of hydroxytyrosol from glucose-induced Insulin deficient and pancreas and liver toxicity in vitro

Khaled Hamdena*, Nourreddine alloucheb, Mohamed Damakb, Serge CarreauC and Abdelfattah Elfeki

aAnimal Ecophysiology, Faculty of Sciences, Sfax, Tunisia
bChemistry of Natural substances laboratory, Faculty of Sciences, Sfax, Tunisia
CUSC 2006 INRA-EA 2608, Biochemistry-University of Caen, France.
*For correspondence - khaled.hamden@yahoo.fr

Keywords: Hydroxytyrosol, Pancreas, Glucose toxicity, Insulin, Antioxidant, Liver slices.

Abstract
The present study aims to investigate the effect of hydroxytyrosol in insulin secretion and the antioxidant activity in liver slices in vitro. For this, pancreas and liver slices were incubated in presence of 1g/l or 4g/l glucose (± hydroxytyrosol (HT)) during 40 minutes. We interest to evaluate the action of HT in insulin secretion, antioxidant enzymes activities (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)), reduced glutathione (GSH), lipid peroxidation, lactate dehydrogenase (LDH) and histological changes in pancreas and liver slices. For the first time, our results show that hydroxytyrosol significantly induces insulin secretion in pancreas incubation. Besides, the present work prove that HT has a good antioxidant activity by preventing the decrease of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) activities and the reducing glutathione content (GSH) in hepatic slices incubated in high glucose concentration (HG) (4g/l). En parallel, a significant decrease in lipid peroxidation rate and lactate dehydrogenase (LDH) activity are observed after hydroxytyrosol administration in liver slices. Theses beneficial actions of HT are confirmed by histological changes in hepatic and pancreatic tissues. Conclusion: HT supplementation in diabetic can induces insulin secretion and prevents glucose toxicity in pancreas and liver.

Introduction
Diabetes mellitus (DM) is a major cause of disability and hospitalization that presents a significant burden on societies worldwide (1,2). Higher glucose level in vitro as well in vivo is the principal sources of reactive oxygen species (ROS) which are play a key role in the apparition of many diseases and disorders (3,4). The generation of ROS has been implicated in the pathogenesis of several forms of acute cell injury, where the oxidative stress process plays a central role (5,6). Oxidative stress is now recognized to be associated with more than 200 diseases (7). Dietary intake of foods rich in antioxidants, as phenolic compounds, is associated with the prevention of cardiovascular disease and reduces risk of liver dysfunction (5) and neuroprotective and cardioprotective actions (8-10). There is a continuing interest to define the preventive effects of phenols against reactive oxygen species mediated degenerative diseases. Phenolic compounds are important bioactive biomolecules that are of increasingly interest for their ability to exert antioxidant actions. A significant number of reports exist in the literature indicating that hydroxytyrosol, a natural exist in olive oil, exerts antioxidative actions which are effective in preventing or reducing the deleterious effects of oxygen-derived free radicals associated with
many diseases (11, 12). In fact hydroxytyrosol has various biological activities, such as, preventing human erythrocytes from oxidative damage induced by hydrogen peroxide in rats (13), and human (14), inhibiting LDL oxidation in vitro, and anti-inflammatory (15-17).

Materials and Methods

**Chromatographic purification of hydroxytyrosol**

Fresh olive mill wastewaters (OMW) were supplied by discontinuous three-phase olive processing mill from a cooperative in Sfax (Tunisia). This sample was generated from Chemlali olive variety. Hydroxytyrosol was purified from OMW as described previously (18). Briefly, the polyphenolic fraction was extracted from OMW using ethyl acetate as solvent. The organic extract was evaporated under reduced pressure below 45 °C. an aliquot (1 g) of the obtained residue was chromatographed on a C-18 silica gel (liChroprep RP-18; 25–40 \( \mu \)m) column (2.5 x 70 cm) under medium pressure. Phenolic compound elution was carried out with a mixture of water/acetonitrile (8:2, V:V). The flow rate was adjusted to 0.3 ml/min and 4.5 ml fractions were collected. These fractions were measured by optical density at 280 nm and the chromatogram (optical density versus fraction number) was represented (data not shown). The first separated peak corresponds to pure hydroxytyrosol.

**Preparation of Liver Slices**

Male adult Wister rat, weighing 280–200 g was taken and dissected after cervical dislocation. The liver and pancreas were removed, transferred and then incubated in KRB buffer (pH 7.4): 118 mM NaCl, 4 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 1.25 g/l BSA and 40 g/l dextran T70. The perfusate was continuously gassed with a mixture of O2:CO2 (95:5). Liver was sliced into small pieces (4–6 mg) of about 0.5 x 0.5 x 0.5 mm using a prep blade. Slices were divided into small portions (20–22 slices) of 100–120 mg wet weight. 1 g liver slices and 1 g pancreas were incubated for 40 min in 5 ml KRB medium at 37 °C equilibrated with 95% O2 + 5%CO2 gas.

**Experiment Design**

1 g liver slices and 1 g pancreas were divided in to three sets: set 1: control, tissues incubated in KRB medium a final concentration of glucose 1g/l; set 2: tissues incubated in KRB medium a final concentration of glucose 4g/l; set 3: slices incubated in KRB medium a final concentration of glucose 4g/l + 50 \( \mu \)g/ml Hydroxytyrosol. Triplicate cultures were set up for each concentration to minimize the errors. Effluent was fractionally collected every 10 minute, and its insulin concentration was measured by a radioimmunoassay kit for Bi-insulin RIA Diagnostic, Pasteur, Paris, France.

After the end of incubation, liver slices were homogenized in ice-cold 100 mM potassium phosphate buffer (pH 7.4) and centrifuged at 4,000xg for 15 min at 4 °C. Liver marker enzyme lactate dehydrogenase (LDH) and antioxidant enzymes SOD, CAT, and glutathione peroxidase (GPx) were estimated in the supernatant. Lipid peroxidation and glutathione (GSH) contents were also measured. For histological studies, pieces of pancreas and liver were fixed in a Bouin Hollande solution for 24 hours, and then embedded in paraffin. Sections of 5\( \mu \)m thickness were stained with hematoxylin-eosin and examined under the Olympus CX41 light microscope.

**Biochemical assays**

Lipid peroxidation was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) using the methods of Buege and Aust (19). In brief, 0.1 ml of liver slices was treated with 2 ml of TBA–
Anti-diabetic and antioxidant effects of Hydroxytyrosol in pancreas and slices liver incubation.

trichloroacetic acid–HCl reagent (0.37%TBA, 0.25MHCl and 15%TCA, 1:1:1 ratio), placed for 15 min in a water bath and then cooled and centrifuged at 3500 × g for 10 min at room temperature. The absorbance of supernatant was measured at 535 nm against a reference blank. Values were expressed as mM/mg protein. Catalase (CAT) was estimated by the method of Aebi (20). The reaction mixture contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml sperm medium and 0.4 ml of 50M H2O2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was readded at 620 nm; CAT activity was expressed as µM of H2O2 consumed/min/mg protein. SOD was assayed according to the technique of Marklund and Marklund (21) based on the inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate, and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of nitroblue tetrazolium reduction per min/mg protein. GPx activity was measured by the method described by Pagila and Valentine (22). Briefly, the reaction mixture contained 0.2 mL of 0.4 M phosphate buffer (pH 7.0), 0.1 mL of 10 mM sodium azide, 0.2 mL of sperm medium (supernatant; homogenized in 0.4 M phosphate buffer, pH 7.0), 0.2 mL of GSH, and 0.1 mL of 0.2 mM hydrogen peroxide. The contents were incubated at 37°C for 10 min. The reaction was stopped by adding 0.4 mL of 10% TCA and centrifuged. The GSH content was estimated in the liver slices by the method of Ellman (23). Liver pieces were lysed with 0.1% EDTA solution and precipitating reagent which contains 0.16% metaphosphoric acid, 0.02% EDTA and 3% NaCl. After mixing, the solution was allowed to stand for 5 min before being filtered. Two milliliter of filtrate was added to 4 ml of disodium hydrogen phosphate (0.1 M, pH 8.0) and 1 ml of DTNB reagent. A blank was prepared from 1.2 ml of precipitating reagent, 0.8 ml of EDTA solution, 4 ml of disodium hydrogen phosphate and 1 ml of DTNB reagent. The color was immediately readded at 412 nm with the help of spectrophotometer. The amount of proteins was determined by the method of Lowry et al (24) using bovine serum albumin as the standard at 660 nm. The activity of lactate dehydrogenase (LDH) and Glycogen content were assayed using commercial kits from Biomaghreb, Tunis, Tunisia. For histological studies, pieces of pancreas and liver were fixed in a bouine’s solution for 24 hrs and then embedded in a paraffin section of 5µm thickness were stained with hematoxylen, eosin and examined under olumpus CX41 light Microscope.

Statistical analysis

Data are presented as means ± SD. The determinations were performed from 6 animals per group and the differences were examined by the one-way analysis of variance (ANOVA) followed by the Fisher test (Stat View) and the significance was accepted at p<0.05.

Results

Antioxidant activity in vitro

In vitro, the antioxidant activity of HT was evaluated by its ability to scavenge DPPH free radicals. HT showed a scavenging activity with a percentage decrease, versus the absorbance of DPPH standard solution of 96% at a concentration of 40 µg/ml (Fig. 1).

Hydroxytyrosol, insulin and pancreas in vitro

Fig. 2 shows that higher glucose concentration causes a decline in insulin secretion after 40 min of pancreas incubation. However, in pancreas incubated at same time with HG concentration and hydroxytyrosol, we investigated in the first time a significant increase in the insulin secretion.
Hydroxytyrosol, antioxidant enzymes and liver in vitro

Fig. 3 shows that HT exert a good antioxidant activity. In fact, this study shows a decrease in the SOD, CAT and GPX activities incubated in higher glucose level (4g/l). Moreover, glucose reduces the GSH contents in hepatic slices. However, in slices liver co-incubated with glucose (4g/l) and HT (50 µg/ml), a good antioxidant action is observed. Hydroxytyrosol in slices liver increase significantly the SOD, CAT and GPX activities and GSH level (Fig3).

Hydroxytyrosol, lipid peroxidation level and lactate deshydrogenase activity and liver in vitro

The effects of HT on the LDH activity and lipid peroxidation level in liver slices homogenate are presented in Fig. 4. LDH and TBARs contents were significantly increased in the liver slice treated with glucose (4g/l) compared to control (1g/l). However, HT administration to incubation medium decrease significantly the two indices contents in liver slices.

Hydroxytyrosol, histological changes and pancreas

Fig. 5 reveals the toxicity of glucose at (4g/l) in incubated pancreas. β cells of Pancreas incubated with glucose (4g/l) during 40 min show damage evidences by intense DNA stained indicator of apoptosis. However, the incubation of pancreas at same time with HT and Glucose, a clearly protective action was observed.

Discussion

Higher glucose concentration in vivo as in vitro favours the formation of advanced glycation endproducts (AGES) (25). The chemical modification of sugars (glycation of proteins) in liver slices causes alteration in the structure and function of tissue proteins such as SOD, CAT, GPX and GSH leads to decrease in the antioxidant capacity. Moreover, the increase of glucose level in culture medium favours glucose auto-oxidation reaction and this induces free radical generation en agreement with others works (26) lead to increase in LDH and TBARS rates. Both the disturbance in the antioxidant capacity and the increase in free radicals by glucose auto-oxidation are associated with excessive concentrations of reactive oxygen species (ROS) which attack many tissues, particularly pancreas; tissue characterised by low

Khaled Hamden et al
Fig. 3. Effect of hydroxytyrosol and glucose (1g/l or 4g/l) on SOD, CAT and GPX activities in liver slices in vitro. Statistical analysis as figure1.

Fig. 4. Time course of thiobarbituric acid reactive substances (TBARS) level and LDH activity in liver slices incubated in medium contain glucose (1g/l); glucose (4g/l) or at same time glucose (4g/l) + hydroxytyrosol (50µg/ml). Statistical analysis as fig. 1.

Anti-diabetic and antioxidant effects of Hydroxytyrosol in pancreas and slices liver incubation.
result illustrates the good role of HT in the protection of pancreatic cells from glucose induces damage and death (Fig. 4); ii) like others herbs extract, HT can enhance insulin secretion by its insulinotropic effects: HT inhibits KATP channels and increases the voltage-dependent calcium channel which plays a key role in insulin secretion (28) iii) the three hydroxyl groups of HT inhibited some intestinal enzymes such as aldose reductase and disaccharidases and this lowered glucose absorption in intestine (29). Besides, in this study, an antioxidant effect of HT was observed in hepatic slices culture. In fact a protective effect of HT against glycation of protein and/or oxidation of glucose is evidenced by significant increase of SOD, CAT and GPX activities in liver slices after HT supplementation. These results are in agreement with several studies (30-32) which have
demonstrated that HT possesses a clear antioxidant property in vitro as in human. HT plays the role of chain breakers or radical scavengers depending on its chemical structure. In fact the antioxidant activity of HT depends on the position of three hydroxyl groups in the molecule. These groups make it of a potential scavenging free radicals activity. This is confirmed by our observation that HT neutralised the DPPH free radicals a lower dose. Moreover, our results show that HT counteracted the oxidative modifications by its capacity of decreasing the TBARs level in hepatic cells in agreement with others (30, 33).

The present study presents interesting results from the time-course determination of GSH and Glucose concentration. In hepatic slices treated only with higher glucose concentration, GSH concentration decreases probably due to both increase of ROS production and the contemporary inactivation of GSH-related enzymes, especially GPX. However, HT administration to slices medium protects from GSH level decrease and this is probably the result of the protection of GPX activity, enzyme able to preserve the functionality of GSH cycle after 40 min. The preservation of GPX activity consequently maintains of the normal content of GSH. Theses results are in accordance with many literature data indicating the potent ‘in vitro’ antioxidant activity of hydroxytyrosol (34,35). This antioxidant activity of HT protects hepatic cells from death and damage by lower contents in LDH, TBARs level and inhibits histological changes compared to liver slices treated with only HG.

In conclusion, this study demonstrated the beneficial effect of HT as an effective hypoglycemic and antioxidant agent in alleviating oxidative stress and free radicals as well as in enhancing insulin secretion and both enzymatic and nonenzymatic defenses diabetes.

Acknowledgements
This work was supported by the Tunisian Ministry of High Education and Scientific Research and Technology and Tunisian Ministry of Public Health.

References


Anti-diabetic and antioxidant effects of Hydroxytyrosol in pancreas and slices liver incubation.


33. González-Santiago M, Martín-Bautista E, Carrero JJ, Fonollá J, Baró L, Bartolomé MV, Gil-Loyzaga P, López-Huertas E. One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces
atherosclerosis development. Therosclerosis. 188:35–42.
