American Journal of Biomedical and Life Sciences
2020; 8(6): 220-224
http://www.sciencepublishinggroup.com/j/ajbls
doi: 10.11648/j.ajbls.20200806.15
ISSN: 2330-8818 (Print); ISSN: 2330-880X (Online)

Effect of the Plant Flavonoid Luteolin on a Mitochondrial Function in the Streptozotocin-induced Diabetic Rats

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To cite this article:
Pozilov Mamurjon Komiljonovich, Ernazarov Zafar Mamurovich, Afzalova Sayyora Abdulahadovna, Asrarov Muzaffar Islamovich, Ergashev Nurali Azamovich, Komilov Baxrom Jamoldinovich. Effect of the Plant Flavonoid Luteolin on a Mitochondrial Function in the Streptozotocin-induced Diabetic Rats. *American Journal of Biomedical and Life Sciences.* Vol. 8, No. 6, 2020, pp. 220-224.
doi: 10.11648/j.ajbls.20200806.15

Received: November 11, 2020; **Accepted**: November 24, 2020; **Published**: December 4, 2020

Abstract: The state of the mitochondrial megapore (mitochondrial permeability transition pore-mPTP), respiration and oxidative phosphorylation of rat liver and pancreas mitochondria in streptozotocin (STZ) - induced diabetes were studied, considered the ways of correction of the detected membrane damage with the flavone luteolin isolated from the plant *Inula caspica*. It was shown that, under conditions of experimental diabetes mellitus, the rate of swelling of rat liver and pancreas mitochondria is higher than of the healthy ones; this means that mPTP of rat liver and pancreas mitochondria is in the open state in pathology. Luteolin recovers mPTP to the normal condition, thus removing the effect of STZ on mitochondria. It was also shown that, the respiration rate of liver and pancreatic mitochondria in the state 3 and state 4 states increases in STZ - induced diabetes, which significantly reduces the respiratory control (RC) and ADP/O coefficients in comparison with the control. The data obtained indicate the disconnection of respiration and oxidative phosphorylation in STZ - induced diabetes. Luteolin (oral dose is 50 mg/kg of body weight, during 8 days) eliminates the detected functional disorders of rat liver and pancreas mitochondria, probably due to its antioxidant properties.

Keywords: Liver, Pancreas, Mitochondria, mPTP, Lipid Peroxidation, Streptozotocin-induced Diabetes, Luteolin, Oxidative Phosphorylation

1. Introduction

Study of cells' structural and functional damage mechanisms in pathology and methods of correcting these injuries with the help of pharmacological agents is a priority of modern endocrinology. In spite of the variety of means for diabetes mellitus treatment, the search for novel pharmacological targets in demand. In the cell, such "targets" are mitochondrial membranes and structures localized in them, primarily the respiratory chain and mPTP) [1, 2]. The role of mPTP is being actively discussed in the development of various pathologies, in particular, diabetes [3]. The formation of reactive oxygen species in the cell and the excessive activation of free radical oxidation processes underlie the development of diabetes mellitus [4, 5]. These processes are considered as a universal mechanism that unites the main biochemical pathways of the toxic effect of hyperglycemia on the body. It is also known that the mitochondrial respiratory chain is the main source of reactive oxygen species [6].

On the other hand, exposure to reactive oxygen species converts mPTP into an open state, mitochondria swell and processes of necrosis or apoptosis are initiated [7]. Along with the synthesis of ATP, mitochondria play an important role in normal metabolism: for example, these organelles

switch pathways leading to the secretion of insulin by β-cells in response to glucose entering the cell [8].

Luteolin is used in traditional medicine for the treatment of various diseases due to the presence of this compound cytoprotective, capillary-strengthening, cancer-preventive, antibacterial, antiviral, immunomodulatory, anti-inflammatory and anti-allergic properties like some other flavones. This flavone has an antioxidant effect, i.e. the ability to bind free radicals, like other flavonoids.

Molecular mechanisms of luteolin interaction with mitochondrial membranes have not been completely established, although it can be assumed that they underlie its pharmacological effect. In the light of the foregoing, the study of the inhibitory effect of plant polyphenols in including luteolin, on the processes of free radical oxidation, uncontrolled lipid peroxidation (LPO), as well as energy supply of cells and the state of mPTP seems to be quite timely and relevant. These studies will help to elucidate the mechanisms of the pharmacological action of flavones and identify original compounds that are promising for the creation of highly effective drugs.

The aim of this work was to study the mechanisms of damage to mPTP, the respiratory and phosphorylating functions of liver and pancreatic mitochondria in STZ-induced diabetes, as well as the possibility of correcting the detected disorders with the help of luteolin.

2. Materials and Methods

For screening and detailed study of the mechanism of pharmacological drugs', various experimental models of diabetes mellitus are widely used that created by the administration of STZ (Wako, Japan), etc., which have a cytotoxic effect on pancreatic β-cells. In the present work, we used a model of experimental diabetes induced by STZ.

2.1. Animals

Experiments were performed on white male rats (180-200 g body weight). The animals were divided into three groups: I group – intact (n=10), II group – the animals with experimental diabetes that once were injected intraperitoneally with an STZ (n=10) (50 mg/kg body weight intraperitoneally in a 0,1 mol/L citrate buffer, pH 4,5) and III group – STZ-induced diabetes+luteolin (n=10) (per os dose of 50 mg/kg of body weight) for 8 days starting from 12 days after administration of STZ and reaching a predetermined level of hyperglycemia.

2.2. Isolation of Mitochondria from Rat Liver and Pancreas

Liver and pancreatic mitochondria were isolated by differential centrifugation [9-10] 12 days after STZ injection, when the blood glucose level reached 11 mmol/L. The composition of the isolation medium for liver mitochondria: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4; for isolation of pancreatic mitochondria, the same isolation medium was used, but it was added bovine serum albumin (BSA) 2 mg/g tissue weight. Mitochondria from both organs were suspended in isolation medium without EDTA.

2.3. Measurement of Mitochondrial Swelling

Ca^{2+}-dependent swelling of mitochondria was recorded by the change in the light diffusion of the mitochondrial suspension (0.5 mg protein/ml) at 540 nm [11].

2.4. Mitochondrial Respiratory Activity

Mitochondrial respiration and oxidative phosphorylation were measured by the polarographic method at 26°C. The assay medium contained 100 mM sucrose, 75 mM KC1, 10 mM Tris-HCl, 2,5 mM K_2HPO_4, pH 7,4 and 10 mM succinate + 2 μM rotenone as respiratory substrates. Protein concentration of mitochondria corresponded to 3 mg/ml of the reaction medium ADP (200 μM) was added as a respiratory stimulant. It was calculated the rate of mitochondrial respiration in different metabolic states: state 3 (V_3) – respiration rate after ADP formation, state 4 (V_4) – respiration rate after spending ADP. The indices characterizing pair of oxidation and phosphorylation in mitochondria: respiratory control (RC) ratio (RC=state 3/state 4) and the coefficient of phosphorylation of ADP/O. Mitochondrial respiration rate in different metabolic states are expressed in nanograms of consumed oxygen atoms per 1 minute per 1 mg of mitochondrial protein. The respiratory control and ADP/O ratio was calculated according to the method of Chance [12].

2.5. Determination of Malondialdehyde Levels in Mitochondria

Lipid peroxidation products was carried out with the participation of thiobarbituric acid (TBA). The reaction was stopped by adding 0,220 ml of 70% trichloroacetic acid in the incubation medium. Thereafter mitochondrial suspensions were centrifuged for 15 minutes at 4000 r/min. Then 2 ml of the supernatant and added 1 ml of 75% solution of TBA. The control tube was poured 2 ml of distilled water and 1 ml of TBA. The mixtures were incubated at 37°C water bath for 30 min. Measure the absorbance at 540 nm after cooling. The quantity of malondialdehyde (MDA) was calculated using a molar extinction coefficient equal to 1.56/10 cm. The rate of LPO reaction expressed in MDA nmol/mg of protein-min.

Blood glucose was determined with the help of glucose oxidase method's set «Glucose - enzymatic-colorimetric test» (Cypress diagnostic, Belgium). The content of mitochondrial protein was determined by the Lowry method in the modification of Peterson [13].

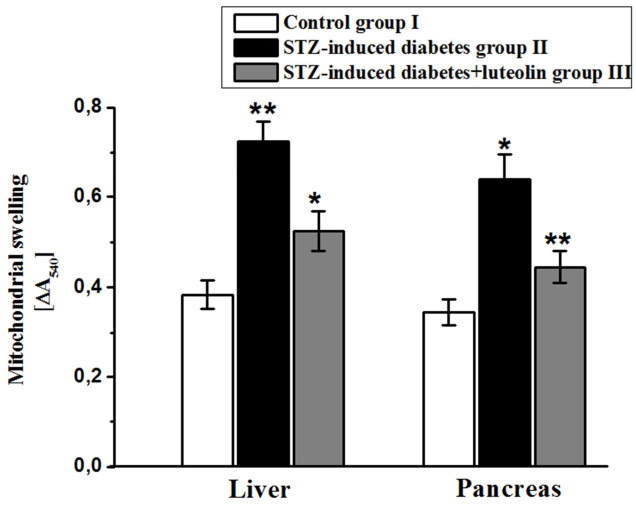
Static analysis of data was performed using the program features Origin 6.1 (OriginLab Corporation, USA). Difference between mean of experimental and control groups were evaluated by unpaired Student's test with $P<0.05$ considered as significant.

3. Result and Discussion

The results of our studies on models of STZ-induced diabetes mellitus in rats showed a significant hypoglycemic

effect after oral administration of luteolin. Figure 1 shows the results of experiments on the study of effects of experimental diabetes and luteolin on the permeability of rat liver and pancreatic mitochondria. Under the experimental conditions we used (the incubation medium contained a Ca^{2+}-EGTA buffer), the swelling of mitochondria can be considered as a result of the open state of mPTP and the suppression of swelling, as closed, i.e. using this technique, it is possible to assess the state of mPTP in STZ-induced diabetes and the action of luteolin. The addition of 10 μM $CaCl_2$ to the incubation medium leads to swelling of rat liver and pancreatic mitochondria and of rats of group I (Figure 1). The rates of liver and pancreatic mitochondrial swelling were 0,38 ΔA_{540}/10 min and 0,34 ΔA_{540}/10 min, respectively.

Under the same conditions, the swelling rate of isolation of rat liver mitochondria of group II (STZ-induced diabetes) was 0,72 ΔA_{540}/10 min, which is 89,4±6,8% higher than that of the control group (Figure 1). The rate of swelling of pancreatic mitochondria (group II of animals) was 0,64 ΔA_{540}/10 min, which is 88,2±7,5% higher than the control (Figure 1). Since under the used conditions, mitochondrial swelling can be considered as the opening of mPTP, the results obtained indicate that in STZ-induced diabetes, of the liver and pancreas are in an opening state of the mPTP.



*P<0,05; **P<0,01 compared to the control group.

Figure 1. *Changes in the swelling of rat liver and pancreatic mitochondria under conditions of STZ-induced diabetes and during luteolin pharmacotherapy. Incubation medium: sucrose - 200 mM, KH_2PO_4 - 1 mM, succinate - 5 mM, Ca^{2+}-EGTA buffer 20 - μM, Hepes - 20 mM, Tris-HCl - 20 mM, rotenone - 2 μM, oligomycin - 1 μg/ml, pH 7.2.*

Luteolin pharmacotherapy in rats with STZ-induced diabetes led to inhibition of the observed swelling of the liver and pancreatic mitochondria. Thus, the rate of mitochondrial swelling, isolated from the liver of group III rats (STZ-induced diabetes + luteolin) was 0,52 ΔA_{540}/10 min, which is 52,6±4,6% less than the swelling rate of liver mitochondria in group II rats (Figure 1). Similar data were obtained when measuring the rates of swelling of the pancreatic mitochondria. Thus, the swelling rate of pancreatic mitochondria in rats (group III) was 0,44 ΔA_{540}/10 min, which is 58,8±5,0% less than the swelling rate of pancreatic mitochondria in group II rats. Thus, the use of a combined preparation of luteolin under conditions of STZ-induced diabetes significantly inhibited mPTP opening (Figure 1).

Therefore, STZ-induced diabetes causes, in particular, the development of mitochondrial dysfunction, manifested by the opening of mPTP. Pharmacotherapy of rats with STZ-induced diabetes by luteolin corrects mitochondrial dysfunction, effectively affecting mPTP state.

In the next series of experiments, it was studied rats' liver and pancreatic mitochondrial respiratory and oxidative phosphorylating activity with STZ-induced diabetes and exposed to luteolin. In case of intoxication with STZ, the respiration rate of rat liver mitochondria in the state 3 increases by 46.7±3.1% compared to that of liver mitochondria of intact animals (table 1). In addition, in comparison with the control, the respiration rate of mitochondria in the state 4 was increased (by 72.7±5.4%). At the same time, in relation to the normal indicators, the coefficients of RC and ADP/O decrease by 15.1±1.0% and 27.6±3.1%, respectively.

The obtained results indicate the activation of respiration during the succinate oxidation in rat liver mitochondria with experimental diabetes in states V_3 and V_4. It was investigated the effect of luteolin's pharmacotherapy on respiration and oxidative phosphorylation of rat liver mitochondria. The mitochondrial respiration rate, isolated from rat liver III-group (pharmacotherapy luteolin) was lower than the rate of respiration of the rat liver mitochondria II-group. Mitochondrial respiration of rat liver in the group III at state 3 of oxidation succinate inhibited by 39,1±3,1% respectively compared STZ-induced diabetes (table 1). Respiration of rat liver mitochondria in the groups III reduced state at succinate state 4; 63,3±4,7% as compared with the mitochondrial respiration II-group. In terms of luteolin pharmacotherapy, RC coefficient increased the oxidation of succinate to 14,0±1,1%, respectively, compared indicator STZ-induced diabetes. Pharmacotherapy with luteolin index increases ADP/O at 20,2±2,1% in the oxidation of succinate as compared with the II-group rats. The findings suggest that pharmacotherapy with luteolin increases the coupling of oxidation and phosphorylation in mitochondria. The results indicate the activation of the respiratory V_3 and V_4 in the oxidation of substrates liver mitochondria STZ-induced diabetes rat, which is partially removed luteolin pharmacotherapy

Identical data were obtained when studying the effect of luteolin on respiration and phosphorylation of pancreatic mitochondria. Experiments have shown that against STZ, the respiration rate of the mitochondria of the pancreas in the state 3 in comparison with state 3 of the mitochondria of the intact group is increased by 30,2±2,3%. With STZ-induced diabetes, the respiration rate of the pancreatic mitochondria in the state 4 also increases by 96,5±6,7% compared to the control, and the RC and ADP/O ratios decrease by 33,8±2,1% and 20,5±2,0%, respectively.

The corrective effect of luteolin in experiments with pancreatic mitochondria is expressed in the restoration of the uncoupled state, i.e. during pharmacotherapy with the drug,

the RC and ADP/O values are normalized: the RC value is only 22,1±1,8% lower than that of the intact group, and the phosphorylation efficiency increases to 14,0±1,2% of the normal value.

Table 1. *Effect of luteolin on respiration and oxidative phosphorylation of rat liver and pancreatic mitochondria with STZ-induced diabetes.*

Experimental conditions	Respiration rate, ng-atom O/min•mg protein		RC	ADP/O
	State 3	State 4		
Liver mitochondria				
Control (intact group)	92,0±2,7	33,0±1,7	2,78±0,20	1,88±0,06
STZ-induced diabetes	135,0±3,3**	57,0±2,4**	2,36±0,15	1,36±0,07**
STZ-induced diabetes+luteolin	99,0±3,0	36,1±2,5	2,75±0,16	1,74±0,05**
Pancreatic mitochondria				
Control (intact group)	79,1±2,6	29,0±2,9	2,72±0,17	1,66±0,09
STZ-induced diabetes	103,0±3,0*	57,0±3,1**	1,80±0,14*	1,32±0,05**
STZ-induced diabetes+luteolin	84,0±3,2**	35,0±2,0**	2,40±0,11	1,55±0,04**

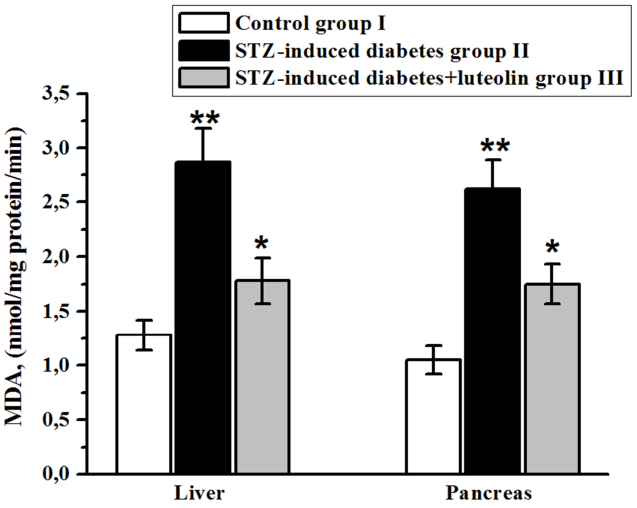
Incubation medium: sucrose - 100 mM, KCl - 75 mM, KH_2PO_4 - 2.5 mM, Tris-HCl - 10 mM, succinate - 10 mM, rotenone - 2 μM, pH-7.4; mitochondrial protein concentration 3.5 mg/ml.
*P<0,05; **P<0,01 compared to the control group.

The results of our experiments indicate the uncoupling of oxidative phosphorylation in liver and pancreatic mitochondria in STZ-induced diabetes, with ATP deficiency in rat tissues and the transition of mPTP to the open state, i.e. it is observed permeability of mitochondrial membranes. Luteolin reduces the effect of STZ-induced diabetes on mitochondrial dysfunction.

Previously, it was shown that intoxication with STZ increases the rate of LPO in the liver mitochondria, causes a hypercompensated low-energy shift with an increase in respiratory rates in all metabolic states and uncoupling of oxidative phosphorylation. Therapy with glycorazmulin and salvifolin normalized the processes of succinate-dependent energy production in mitochondria, with the restoration of the uncoupling of oxidation and phosphorylation, and decreased LPO in the liver [14, 15].

It was studied effect of biological active flavone - luteolin on LPO processes in liver and pancreatic mitochondria in STZ-induced diabetic rats in *in vivo* experiments. Obtained results indicate an increase of MDA amount by 124,2±9,7% and 149,5±12,2% in liver and pancreatic mitochondria in STZ-induced diabetic rats (II group) compared to control (I group), respectively (Figure 2).

Pharmacotherapy of STZ-induced diabetic rats using luteolin caused a reduction in the amount of MDA in mitochondria of liver and pancreas: action of luteolin by 85,1±6,4% and 82,8±5,3% in comparison with animals of STZ-induced diabetic II group, respectively (Figure 2). Thus, substances luteolin strengthened mitochondrial effectively inhibited LPO and antioxidant system in liver and pancreas at STZ-induced diabetes. Under the experimental conditions that we used, apparently the generation of reactive oxygen species under the action of STZ accelerates LPO processes in mitochondrial membranes, because of which mPTP goes into an open state, respiration and are uncoupled oxidative phosphorylation. Realizing its antioxidant properties, luteolin prevents the formation of lipid peroxides, stabilizes mitochondrial membranes and thus restores their bioenergy functions, which improves not only energy metabolism, but also cellular functions.



*P<0,05; **P<0,01 compared to the control group.

Figure 2. *Effect of luteolin on LPO intensity in rat liver and pancreatic mitochondria in STZ-induced diabetes.*

4. Conclusions

1. Under STZ-induced diabetes' conditions, the liver and pancreatic mPTP becomes open that may be one of the mechanisms of damage to mitochondrial function, as well as cells in experimental diabetes.
2. In STZ-induced diabetes, it is observed increase in respiration rates in the V_3 and V_4 states, leading to uncoupling of oxidative phosphorylation and an ATP deficiency in the mitochondria of the liver and pancreas in rat tissues.
3. For the first time it was revealed new hypoglycemic properties of luteolin. At STZ-induced diabetes luteolin effectively inhibit LPO in mitochondrial membranes of liver and pancreas.

The work was performed within the framework of the grant for fundamental research of the Academy of Sciences of the Republic of Uzbekistan №. FA-F5-T084.

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