Gaziosmanpaşa Üniversitesi Tıp Fakültesi Dergisi 2023;15(1):26-44 Araştırma Makalesi

Roziglitazonun Sıçanlarda Mitokondriyal Genom Üzerindeki Etkisinin Moleküler Yöntemlerle Araştırılması

Investigation of Effect of Rosiglitazone on the Mitochondrial Genome in Rats by Molecular Methods

Zeliha Cansel Özmen[1], Muhlise Alvur[2], Abdulkerim Bedir[2], Birsen Bilgici[2], Rukiye Nar[3]

[1]Tokat Gaziosmanpasa University Faculty of Medical School, Department of Medical Biochemistry

[2]Ondokuz Mayıs University Faculty of Medical School, Department of Medical Biochemistry

[3]Pamukkale University Faculty of Medical School, Department of Medical Biochemistry

Correspondance Author:

Adress: Assistant Professor Zeliha Cansel Ozmen, University of Tokat Gaziosmanpasa, Faculty of Medicine, Department of Medical Biochemistry, 60100 Tokat, Turkey.

Phone number : +90356 212 95 00/ 7115

fax number:+90 356 213 31 79

e-mail:zcozmen@gmail.com

Özet

Amaç: Roziglitazon, Tip II diabetes mellitus tedavisinde kullanılan tiazolidindion sınıfına ait bir ilaçtır. Bu çalışmanın amacı, farklı sıçan dokularında mtDNA^{4834} delesyon frekansını inceleyerek, roziglitazonun mitokondriyal DNA üzerindeki etkilerini araştırmaktır.

Gereç ve Yöntemler: Sprague-Dawley sıçanlarının karaciğer, ince bağırsak, pankreas dokusu ve lenfositlerinden genomik DNA izole edildi. Numunelerin nükleer DNA, mitokondriyal DNA ve mtDNA^{4834} delesyon kopya sayıları real-time qPCR ile belirlendi. Hücre başına mtDNA kopya sayıları (mtDNA/hücre) ve mtDNA^{4834} delesyon frekansı (delesyon frekansı (%) hesaplandı.

Bulgular: mtDNA/hücre, roziglitazonun tüm dozlarında ince bağırsak ve pankreas dokusunda kontrol grubuna göre anlamlı derecede düşüktü ($p<0.05$). Roziglitazon tüm dozlarında karaciğer dokusundaki mtDNA^{4834} delesyon frekansı (%) kontrol grubuna göre anlamlı olarak düşük bulundu ($p<0.05$). Aynı zamanda kontrol grupları arasında mtDNA^{4834} delesyon frekansı (%) ve mtDNA/hücre açısından istatistiksel olarak anlamlı farklılık vardı ($p<0.05$)

Sonuç: Çalışmamızın sonuçları bize, karaciğer dokusunda mtDNA^{4834} delesyon sıklığının azalması nedeniyle roziglitazonun bu doku üzerinde oksidan etkisinin olmadığını düşündürdü. Ancak ince barsak ve pankreas dokularında mitokondriyal DNA kopya sayılarındaki azalma roziglitazonun oksidan etkisinden kaynaklanmış olabilir.

Anahtar Kelimeler: mtDNA^{4834} delesyon, mitokondriyal DNA, oksidatif stres, roziglitazon

Abstract

Objectives: Rosiglitazone is a drug which belongs to tiazolidinedione class used in the treatment of Type II diabetes mellitus. The aim of this study was to investigate the effects of rosiglitazone on the mitochondrial DNA by examining the frequency of the mtDNA^{4834} deletion in the different rat tissues.

Material and Methods: Genomic DNA was extracted from liver, small intestine, pancreas tissue and lymphocytes of male Sprague-Dawley rats. Nuclear DNA, mitochondrial DNA and mtDNA^{4834} deletion copy numbers of the samples was determined by real-time qPCR. mtDNA copy numbers per cell (mtDNA/cell) and mtDNA^{4834} deletion frequency (mtDNA^{4834} deletion frequency (%)) were calculation.

Results: The mtDNA/cell in the tissues of small intestine and pancreas were significiantly lower than in all rosiglitazone doses compared to the control group ($p<0.05$). The mtDNA^{4834} deletion frequency (%) in the liver tissue at all doses of rosiglitazone was found to be significiantly lower than in the control group ($p<0.05$). At the same time, there was a significiant statistical difference in the mtDNA^{4834} deletion frequency (%) and mtDNA/cell between the control groups ($p<0.05$)

Conclusion: The results of our study made us think that rosiglitazone has no oxidant effect on this tissue due to the decrease in the frequency of mtDNA^{4834} deletion in liver tissue. However, the decrease in mitochondrial DNA copy numbers in the small intestine and pancreas tissues may be due to the oxidant effect of rosiglitazone.

Key Words: mtDNA^{4834} deletion, mitochondrial DNA, oxidative stress, rosiglitazone

Introduction

Rosiglitazone (RSG), a Thiazolidinediones group (TZDs) is a new class of oral antidiabetic agents that directly target insulin resistance. Drugs in this class act as ligands for the peroxisome proliferator-activated receptor gamma (PPAR γ), which regulates genes that control glucose homeostasis and lipid metabolism (1, 2). Several studies suggest that RSG can reduce oxidative stres, independent of their ability to reduce hyperglycaemia (3-5). The mechanism of its antioxidant effect is unclear. On the other hand, it has been reported that TZDs increased oxidative stres (6-8).

During oxidative phosphorylation, mitochondria consume greater than 90% of the oxygen in a cell, and about 1-3% of the oxygen is released as superoxide and hydrogen peroxide (9). Complex I of the mitochondrial electron transport chain has been widely accepted as a major site of mitochondrial reactive oxygen species (ROS) production (10,11). It has been demonstrated that PPAR-ligands decrease respiratory control, induce uncoupling of the oxidative phosphorylation, and reduce the activity of complex I in cell culture and tissue homogenates (6,7). Also, TZDs have been shown to generate ROS as a result of the modulation of mitochondrial functions in several cell types (12).

Mammalian cells contain a few hundred to several thousand mitochondria, each with 2-10 copies of the genom (13). Mitochondrial DNA (mtDNA) is a double-stranded, circular, 16.5-kb molecule

containing genes necessary for the synthesis of the catalytic components of oxidative phosphorylation. Due to its location near the electron transport chain, the absence of protective histones, and the inability in the DNA repair system, mtDNA is susceptible to oxidative damage (14). Many different types of mtDNA point mutations, duplications and large-scale deletions have been identified. Among various types of mtDNA deletions, a 4977- bp deletion in humans and a 4834-bp deletion (mtDNA^{4834} deletion, common deletion 250) in rodents, are commonly found to accumulate in aging tissues (15,16). The mtDNA ^{4834} is defined by a 4834 bp deletion flanked by 2 16-bp repeats located at nt 8103-8118 (ATP6 gene) and nt 12937-12952 (ND5 gene) in the mitochondrial genome (17). The common deletion is often used as a specific indicator of oxidative stress (18). The aim of this study was to investigate the effects of rosiglitazone on the mitochondrial DNA by examining the frequency of the mtDNA^{4834} deletion in the different rat tissues.

Materials and Methods

DNA isolation from rat tissues

Sprague-Dawley rats were obtained from our breeding colony at Samsun; 16 male rats were used for the experiments at 7–8 weeks of age (weighing 248–275 g). Permission for the animal studies was obtained from the Samsun Ondokuz Mayıs University Ethics Committee, and the experiments were performed according to their guidelines for the care and use of laboratory animals. Rats divided into four groups, four in each group; 2.0 mg/kg, 1.0 mg/kg, 0.5 mg/kg RSG (Cayman Chemical Company Ann Arbor, MI) was administered by oral gavage at the same time every morning for 14 days. The control group was given DMSO (Dimethyl sulfoxide), which is used only for dissolving drugs (3.0% DMSO in PBS).

At the end of 14 days, and 4 hr after the last treatment, all the rats were killed by decapitation under a short ether anesthesia. Blood was collected into tubes containing EDTA by heart puncture. After layering 1.5 mL of blood over 1 mL of Histopaque-1077 (Sigma), rat lymphocytes were isolated by centrifugation at 1500g for 20 min at 4-8 ^{0}C. Liver, small intestine, and pancreatic tissues were excised and removed. Rats tissues and lymphocytes were stored at -70 ^{0}C until analysis. Tissue specimens were pulverized with the liquid nitrogen. Total DNA was extracted from liver, pancreas, small intestine and lymphocytes using the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Germany). DNA concentrations were determined in µg/mL by the nanophotometric absorbance at 260 nm (IMPLEN NanoPhotometer, U.K.).

Oligonucleotide Primers Design

The acidic ribosomal phosphoprotein P0 (36B4) gene was selected to determine the number of nuclear genomes in the samples and to normalize the PCR study. Using the LightCycler Probe Design Software 2.0 program, 36B4 external standard (ES) primers (Genbank Accession No: NW047376.1) that will generate 453 basepairs (bp) long amplicon and 36B4 primers that will generate 80 bp amplicon from 453 bp 36B4 standard sequence (Genbank Accession No: NW047376.1) and D-loop external standard

(ES) primers then will generate 321 bp long amplicon which selected from the mitochondrial genome (Genbank Accession No: X14848) was determined. D-loop primers that have been generated 83 bp amplicon from 321 bp D-loop external standard sequence and common deletion primers were previolusly described by Niclas et al. (19) (Genbank Accession No: X14848). The primers used in this study are summarized in Table 1.

Table 1: Position of primers, amplicon length and primer sequences

Primer	Position	Amplicon (bp)	Primer Sequence
36B4 ES Forward	42317661-42317680	453	5'- GAACAACCCCGCTCTGGAGA- 3'
36B4 ES Reverse	42317228-42317245		5'- GCACATCGCTCTGAGGAA- 3'
36B4 Forward	42317288-42317307	80	5'- TCCGTATTGCAGAGTCCTTG- 3'
36B4 Reverse	42317228-42317245		5'- GCACATCGCTCTGAGGAA- 3'
D-loop ES Forward	15786-15806	321	5'- GGCCATCAATTGGTTCATCGT- 3'
D-loop ES Reverse	16084-16106		5'- GGCATTGAAGTTTCAGGTGTAGG- 3'
D-loop Forward	15772-15793	83	5'- GGTTCTTACTTCAGGGCCATCA- 3'
D-loop Reverse	15831-15854		5'- GATTAGACCCGTTACCATCGAGAT- 3'
mtDNA^{4834} Delesyon Forward	8094-8116	81	5'- AAGGACGAACCTGAGCCCTAATA- 3'
mtDNA^{4834} Delesyon Reverse	12984-13008		5'- CGAAGTAGATGATCCGTATGCTGTA- 3'

ES: External Standard

PCR Methods

To determine copy numbers of nuclear genom and mitochondrial genom standard curves were required. PCR reactions were performed with 36B4 ES primers and D-loop ES primers using DNA isolated from a single control rat. The content of the mastermix prepared for 36B4 ES and D Loop ES PCR and the PCR device (Techne Genius PCR Thermal Cycler, Cambridge Ltd, UK) program for 36B4 ES and D Loop ES were shown in Table 2 and Table 3. PCR products that 453 bp sequence

of the 36B4 gene and 321 bp sequence of the mt D-loop region were purified by High-Pure PCR Product Purification kit (Roche Diagnostics GmbH, Germany) and determined by the spectrophotometric absorbance at 260 nm (IMPLEN NanoPhotometer U.K.). The PCR products were separated on a 3% agarose gel at 80 V for 40 min and were detected under UV transillumination (Avagene, PacificImage Electronics) after ethidium bromide staining (Figure 1).

Table 2: Mastermix for 36B4 ES (453 bp) and D Loop ES (321 bp)

	36B4 ES and D Loop ES
Contents	Amount
36B4 Standart Forward Primer	0,25 µM
36B4 Standart Reverse Primer	0,25 µM
D loop standard Forward Primer	-
D loop standard Reverse Primer	-
Taq Buffer (Roche)	1 X
Taq DNA Polymerase (Roche)	1,25 U/ 50 µL
dNTP (Fermantase)	0,2 mM
MgCl	2.0 mM
template DNA	50 ng/50 µL

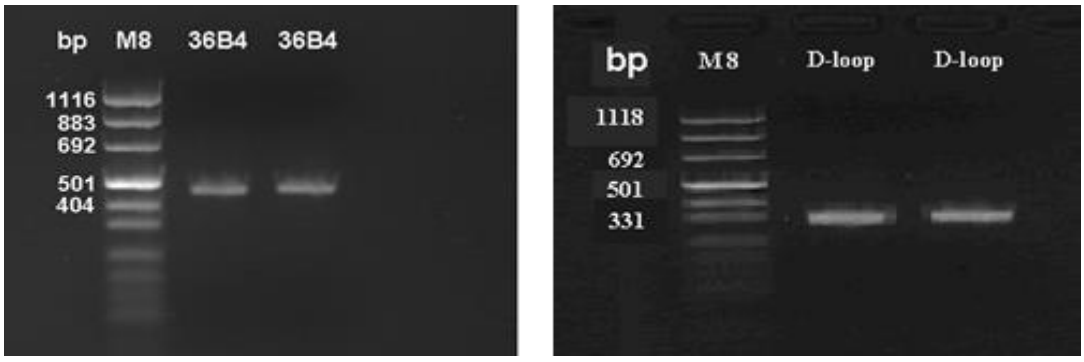

Figure 1: Electrophoretic image of the bands of 36B4 ES and D-loop ES (bp: basepair, M8: Marker 8)

Table 3: PCR program for 36B4 ES (453 bp) and D Loop ES (321 bp)

	36B4 ES and D Loop ES		
Stage	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 min	1
Denaturation	94 ºC	1 min	30
Annealing	60 ºC	30 sec	30
Elongation	72 ºC	1 min	30
Final Elongation	72 ºC	5 min	1
Cooling	6 ºC	4 hour	1

External standard copy numbers were calculated based on the molecular weight of PCR products. Serial dilutions were made, and real-time qPCR reactions were performed with 36B4 primers and D-loop primers by construct the standard curve for the rat 36B4 gene (80 bp) and mitochondrial D-loop region (83 bp) (Table 4 and Table 5). For this purpose, the copy numbers of the nuclear genom and mitochondrial genom in the DNA isolated from a single control rat were calculated from the corresponding this standard curve. This control rat sample was used in our study as a nuclear and mitochondrial genom standards. Then in order to find the nuclear and mtDNA copy numbers, the all samples and the the genom standards were studied with real-time PCR device (LightCycler, Roche Diagnostics GmbH, Mannheim, Germany). Real-time qPCR master mix and real-time qPCR program prepared for the rat genome standard and all samples were shown in Table 4 and Table 5, respectively.

Table 4: Mastermix for 36B4 genom standard, D Loop genom standard and samples

Contents	36B4 standard Final Concentration	D Loop standard Final Concentration
36B4 Standard Forward Primer	0.25 μM	-
36B4 Standard Reverse Primer	0.25 μM	-
D loop Standard Forward Primer	-	0.25 μM
D loop Standard Reverse Primer		0.25 μM
SYBR Green (Sigma-Aldrich)	0.2X	0.2X
LightCycler-FastStart DNA Master Hybridization Probes (Roche)	0,5 U/ 20 μL	0,5 U/ 20 μL
MgCl	3.0 mM	3.0 mM
UNG (Roche)	-	0,5 U/ 20 μL

UNG (Uracil-N-Glycosylase)

Table 5: Real-time PCR program for 36B4 genom standard, D Loop genom standard and samples

Stage	36B4 genom standard			D Loop genom standard		
	Temperature	Time	Cycles	Temperature	Time	Cycles
UNG decontamination	-	-	-	30 ºC	5 minute	1
Initial denaturation	95 ºC	10 minute	1	95 ºC	10 minute	1
Denaturation	95 ºC	5 second	40	95 ºC	5 second	45
Annealing	56 ºC	10 second	40	56 ºC	10 second	45
Elongation	72 ºC	6 second	40	72 ºC	6 second	45
Cooling	6 ºC	4 hour	1	6 ºC	4 hour	1

The 81 bp mtDNA^{4834} deletion amplicon to be used as a ES standard with the PCR device was amplified using mtDNA^{4834} deletion primers. As a sample, a rat genome was used. Used in PCR the master mix and PCR program was shown in Table 6 and Table 7. The PCR products were separated on a 3% agarose gel at 80 V for 40 min and were detected under UV transillumination after ethidium bromide staining (Figure 2).

Table 6: Mastermix for mtDNA^{4834} deletion ES standard

Contents	mtDNA^{4834} deletion Final Concentration
mtDNA^{4834} Deletion Standard Forward Primer	0.25 µM
mtDNA^{4834} Deletion Standard Reverse Primer	0.25 µM
Taq Buffer	1X
$MgCl_2$	2.0 mM
Taq DNA Polymerase	1,25 U/ 50 µL
dNTP (Fermentase)	0.2 mM

Table 7: PCR program for mtDNA^{4834} deletion ES standard

	mtDNA^{4834} deletion		
Stage	Temperature	Time	Cycles
Initial denaturation	94 ºC	4 minute	1
Denaturation	94 ºC	45 second	30
Annealing	56 ºC	30 second	30
Elongation	72 ºC	30 second	30
Final Elongation	72 ºC	2 minute	1
Cooling	6 ºC	4 hour	1

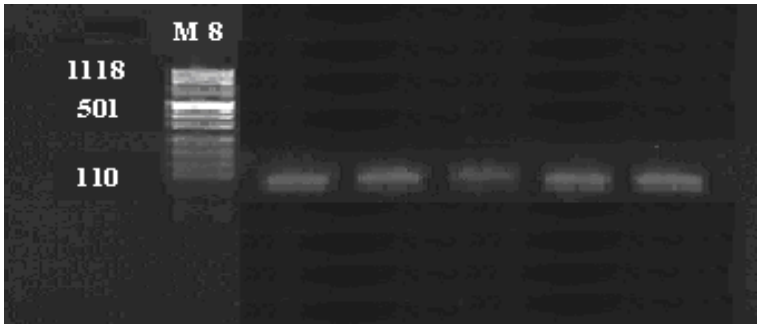

Figure 2: Electrophoretic image of the bands of mtDNA^{4834} Deletion (bp: basepair, M8: Marker 8)

PCR product that 81 bp sequence of the common deletion were purified by SephadexTm G-50 Medium filter (Amersham Biosciences, 100 g) and determined by the spectrophotometric absorbance at 260 nm. Standard DNA for common deletion sequence was generated by cloning the PCR products of the corresponding target sequence into pGEM®-T Easy vector (Promega) and then transforming to the JM109 cells (Promega). Plasmid DNA was extracted and quantified. Them after common deletion standard was determined by the spectrophotometric absorbance at 260 nm. The copy number was calculated based on the molecular weight of the plasmid DNA ($2x10^{10}$ copy/µl.) Then, 7 standards were prepared with 10-fold dilution and the real-time qPCR reactions were performed to construct the standard curve. In order to find the mtDNA^{4834} deletion copy numbers, the all samples and the above standards were studied with the real-time qPCR device (Table 8 and Table 9).

Table 8: Mastermix for mtDNA^{4834} deletion standard and samples

Contents	mtDNA^{4834} deletion Final Concentration
mtDNA^{4834} Deletion Standard Forward Primer	0.25 µM
mtDNA^{4834} Deletion Standard Reverse Primer	0.25 µM
SYBR Green	0.2 X
$MgCl_2$	3.0 mM
LightCycler-FastStart DNA Master Hybridization Probes	0,5 U/ 20 µL
UNG (Roche)	0,5 U/ 20 µL

UNG (Uracil-N-Glycosylase)

Table 9: Real-time PCR program for mtDNA^{4834} deletion standard and samples

	mtDNA^{4834} deletion		
Stage	Temperature	Time	Cycles
UNG decontamination	30 ºC	5 minute	1
Initial denaturation	95 ºC	10 minute	1
Denaturation	95 ºC	5 second	45
Annealing	56 ºC	10 second	45
Elongation	72 ºC	3 second	45
Cooling	6 ºC	4 hour	1

Calculation of mtDNA Copy Numbers Per Cell in Samples (mtDNA/cell)

Nuclear and mitochondrial copy numbers of all samples were found by performing absolute quantitation with qPCR. The D-loop copy numbers of the samples were divided by the 36B4 copy numbers and multiplied by two to calculate the mtDNA/cell.

(mtDNA/cell = (D-loop copy/36B4 copy) x2).

Calculation of mtDNA^{4834} Deletion Frequency in Samples (mtDNA^{4834} deletion frequency (%))

Mitochondria copy numbers and mtDNA^{4834} deletion copy numbers of all samples were determined by absolute quantitation with qPCR. The mtDNA^{4834} deletion copy numbers of the samples were divided by the D-loop copy numbers and multiplied by 100 to calculate the mtDNA^{4834} deletion frequency (%).

(mtDNA^{4834} deletion frequency (%))= (mtDNA^{4834} deletion copy/D-loop copy) x100).

Statistical method

The data were transferred to the SPSS 16.0 (Statistical Package for the Social Sciences) computer package program and statistical evaluations were made. It was determined that the groups did not comply with th

e normal distribution in terms of the compared parameters. The non-parametric Kruskal-Wallis test was used to determine the mtDNA/cell counts and mtDNA^{4834} deletion frequency (%) of tissues and lymphocytes at different RSG doses. In statistical evaluations, the level of significance was accepted as $p< 0.05$.

Results

In order to see the accuracy of the studies, slope and efficiency values obtained from 36B4, D-loop and mtDNA^{4834} deletion PCR studies performed on different dates are shown in Table 10.

The mtDNA/cell and mtDNA^{4834} deletion frequency (%) of rat tissues and lymphocytes obtained after the study were shown in Tables 11 and 12 with their mean, minimum and maximum values. The mtDNA/cell in the tissues of small intestine and pancreas were significiantly lower than in all RSG doses compared to the control group ($p<0.05$). The mtDNA^{4834} deletion frequency (%) in the liver tissue at all doses of RSG was found to be significiantly lower than in the control group ($p<0.05$). mtDNA/cell in small intestine, mtDNA/cell in pancreas and mtDNA^{4834} deletion frequency (%) in liver tissue grafics were shown in Figure 3, 4 and 5 respectively.

Table 10: Slope and efficiency results of 36B4, D-loop and mtDNA^{4834} deletion studies

	36B4		D-loop		mtDNA^{4834} deletion	
	Slope	Efficiency	Slope	Efficiency	Slope	Efficiency
1. Study	-3,589	0,90	-3,439	0,95	-3,554	0,91
2. Study	-3,147	1,078	-3,55	0,91	-3,640	0,88
3. Study	-3,314	1,003	-3,534	0,92	-3,589	0,90
4. Study	-3,060	1,121	-3,379	0,98	-3,843	0,82
5. Study	-3,10	1,101	-3,380	0,98	-3,899	0,80
6. Study	-3,024	1,141	-3,386	0,97	-3,889	0,81
7. Study	-3,145	1,079	-3,395	0,97	-3,77	0,84
8. Study	-3,160	1,072	-3,368	0,98	-3,706	0,86
9. Study					-3,741	0,85
Average	-3,19	1,06	-3,43	0,96	-3,74	0,85

Table 11: The mtDNA/cell in tissues of rat and lymphocyte

Tissues	Control	0,5 mg/kg RSG	1 mg/kg RSG	2 mg/kg RSG
Liver	45451 (35788-62307)	45450 (32228-65541)	47819 (24959-68839)	45100 (21584-91731)
Small intestine	5613 (2669-11583)	2858* (1369-5910)	3321* (2504-5161)	2032* (532-5126)
Pancreas	58229 (30941-91258)	23350* (7684-49053)	16962* (10770-21559)	40617* (10506-85921)
Lymphocyte	25454 (10196-37502)	28294 (12798-42113)	28687 (12463-65209)	23971 (15141-39915)

Kruskal-Wallis Test (Median and Min-Max, * Difference from the control group, $p<0.05$)

Table 12: The mtDNA^{4834} deletion frequency (%) in tissues of rat and lymphocyte

Tissues	Control	0,5 mg/kg RSG	1 mg/kg RSG	2 mg/kg RSG
Liver	4,22 (3.35-4.81)	3,87 (3.14-5.65) *	3,53 (2.42-5.03) *	3,24 (2.38-4.40) *
Small intestine	2,88 (1.66-4.09)	2,77 (2.25-3.23)	2,85 (1.79-4.24)	2,88 (1.99-4.17)
Pancreas	4,77 (4.24-5.36)	4,69 (2.66-6.79)	4,70 (3.62-6.61)	4,66 (3.52-6.24)
Lymphocyte	5,21 (1.96-7.61)	5,73 (4.42-7.24)	5,27 (3.69-7.18)	5,82 (4.82-7.65)

Kruskal-Wallis Test (Median and Min-Max, * Difference from the control group, $p<0.05$)

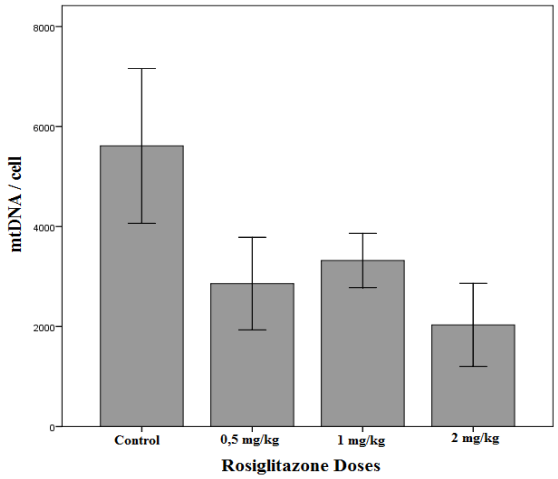

Figure 3: mtDNA/cell in small intestine tissue

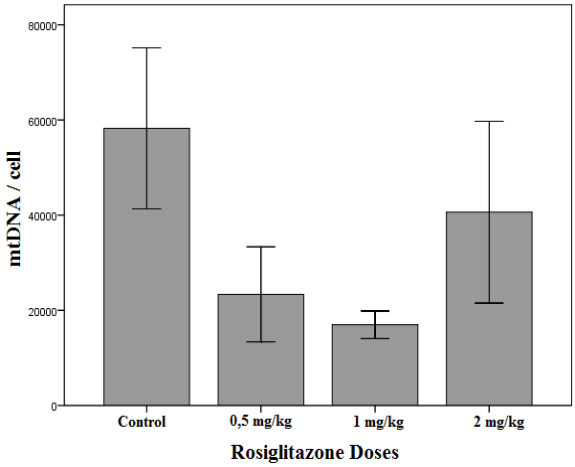

Figure 4: mtDNA/cell in pancreas tissue

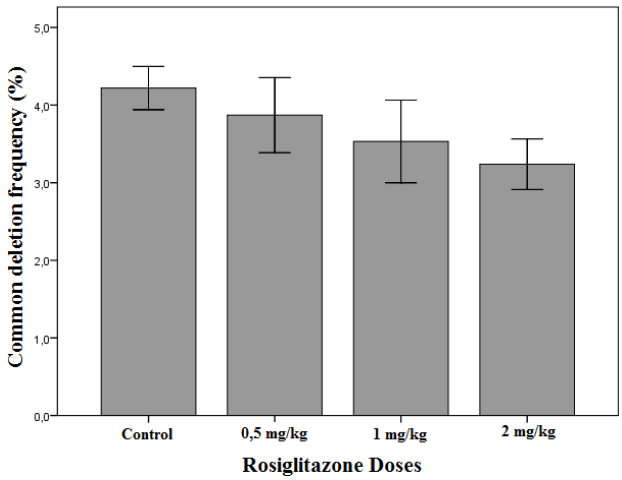

Figure 5: mtDNA^{4834} deletion frequency (%) in liver tissue

At the same time, there was a significiant statistical difference in the mtDNA^{4834} deletion frequency (%) and mtDNA/cell between the control groups ($p<0.05$) mtDNA/cell was found to be lowest in small intestine control tissue and highest in pancreas control tissue. mtDNA^{4834} deletion frequency (%) was found to be lowest in small intestine control tissue and highest in lymphocytes (Figure 6).

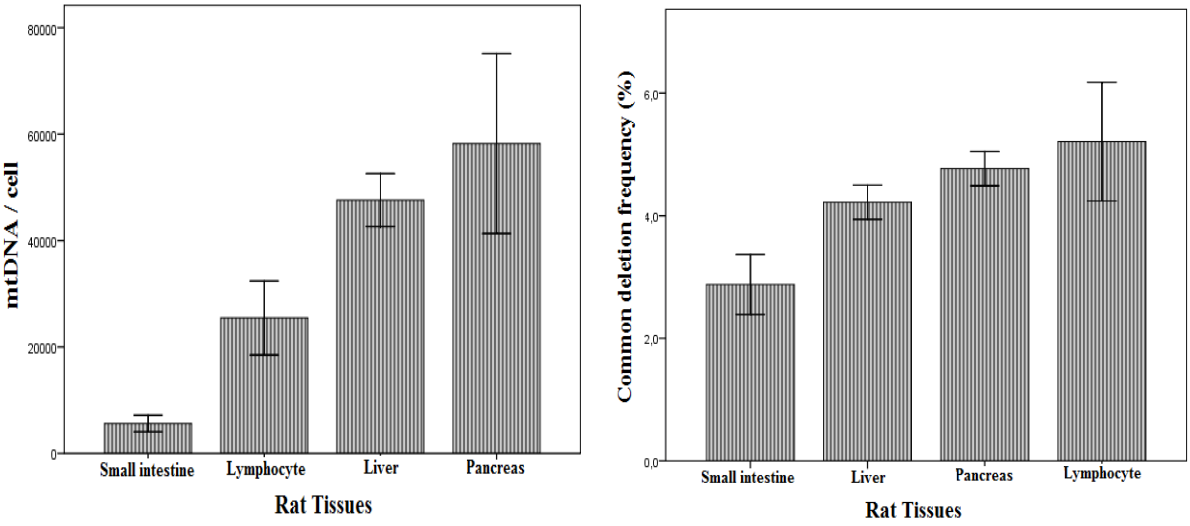

Figure 6: mtDNA/cell and mtDNA^{4834} deletion frequency (%) between the control groups

Discussion

RSG is a TZD group drug used for the treatment of Type II diabetes, which shows its effect by binding to the PPARγ receptor (20). It is considered that, various PPARγ ligands induces production of oxidants and the toxic effect of troglitazone against the rat hepatoma cells may be related to these oxidants (12). Bedir et al. showed the dose-dependent genotoxicity of RSG against the rat liver tissue and lymphocytes, by using single-cell gel electrophoresis (Comet) procedure (21). Also in another trial, in which their used the same procedure, shown that pioglitazone was genotoxic for the rat liver and lymphocytes, by using FPG and Endo III DNA repair enzymes found that the reason of this toxicity was oxide purines and pyrimidines that occur in genomic DNA (22). In a study in obese mice, they showed that, by inhibiting the complex I activity of RSG, it increased ROU formation and caused oxidative stress (23). Besides, they reported that short time TZD administration can increase oxidative stress (24) and TZDs can affect the mitochondria by more than one mechanism (20).

In contrast to these studies, illustrated that TZDs can reduce oxidative stress, and this was independent from the reduction of hyperglycemia as a property of

TZDs (3). It showed that the oxidative stress that induced by glucose in endothelial cells prevented by RSG and this mechanism was independent from PPARγ, it related to activation of 5' AMP activated protein kinase (AMPK) (25). The mechanism of TZD group drugs' antioxidant effects is undetermined. It submitted that hydroxylation of pridins and phenyl circles, which are in the chemical structure of RSG, facilitates purifying of hydroxyl radicals (26). Showed that RSG increases the catalase enzyme activity in the rat brain microvascular endothelial cells (RBMEC), as dose-dependent. In addition, it reported that SOD and catalase enzyme promoters include PPRE, therefore expression of these enzymes modulated by RSG and RSG prevents the effect of ROS that occurs (27).

Shown that oxidative stress cause to deletion or loss of function due to mtDNA base modifications (28). It has been reported that mitochondrial respiratory functions may decrease in tissues with mtDNA deletion (29) and ROU may increase with decreased ATP production (30). Studies have shown that $mtDNA^{4834}$ deletions are associated with aging and aging-related diseases because of decreased oxidative phosphorylation capacity (31). In studies in which competitive PCR used, as reported that, in the older rat tissues, the amount of $mtDNA^{4834}$ deletion was higher than in the young rat tissues. $mtDNA^{4834}$ deletion was reported in liver as % 0.0188 (32), % 0.02 (17), % 1.17 (33) and % 0.5 (18). Similarly, $mtDNA^{4834}$ deletion established in the pancreas as % 0.0639 and in the intestinal mucosa as % 0.0152 (33). In a study in which the relative QPCR method used, the $mtDNA^{4834}$ deletion frequency was % 0.18 in young rat liver tissue, and % 0.59 in older rat liver tissues (19). In this study in which we used QPCR, at the young rats that were not given RSG, we found the $mtDNA^{4834}$ deletion frequency as % 4.22 in liver, % 2,88 in intestines, % 4.77 in pancreas and % 5.21 in lymphocytes. Any study has not published about investigating the effect of RSG on $mtDNA^{4834}$ deletion by plasmid cloning. On the contrary competitive PCR and the relative PCR method we estimated the deletion frequency of our samples by using absolute standard curve and the standard which was got in the plasmid cloning study. The difference between our results and the studies that are defined in literatures may be related to QPCR method that we performed and to the Sprague-Dawley rat kind we used. In the liver tissue, at the all doses of RSG decreased at the $mtDNA^{4834}$ frequency according to the control, we found that this effect didn't occur at the other tissues. We considered that, decreasing of $mtDNA^{4834}$ deletion frequency in liver tissue may related to GPx and SOD enzymes' high activity in the liver (33), and also may be RSG increase expression of these antioxidant enzymes (34).

mDNA copy number presents a variation according to types of cell and tissue, and also to phases of the cell development and differentiation (35). Conducted with competitive PCR, shown that heart, brain and muscle tissue have higher mtDNA than kidney, lung, liver, pancreas, intestinal mucosa and bone marrow (33). During the aging, rising oxidative stress has a critical role in the increase of content and amount of mtDNA (36). RSG increased the number of mtDNA in human adiposis cell culture (37). Supported that, increase of mtDNA content by aging is related to compensation of the mutant DNA by defective mitochondria (38). Contrary to these studies, in another

studies that was performed on human skeletal muscle, a decrease in mtDNA copy. The authors were determined a decrease in mitochondrial function and ATP production by aging (39,40). In a study that was performed on human pancreas islet cells, a decrease in the amount of mtDNA copies was showed in isolated islet cells related to aging. Also, it was found that did not relate this decrease to deletion of mtDNA (41). Gadaleta et al. founded 860 mtDNA copies/cell with competitive PCR in rat liver (17). Nicklas et al. on the contrary, founded a low mtDNA copy number (512 copies/cell) in the rat liver with a relative PCR study, and they attributed this to the pseudogene content of β-actin (19). In our study, we used the pseudogene-free 36B4 single copy gene, which we think is more suitable for a more accurate calculation of mtDNA copy numbers. In this study, we found different mtDNA copy numbers in young rat tissues and lymphocytes, which were not given RSG, as in other studies. We determined mtDNA copy numbers per cell as 45451 in the liver, 5613 in the small intestine, 58229 in pancreas and 25454 in lymphocytes, that this difference between tissues as statistically significant. The reason the mtDNA copy numbers we found are higher than other studies may be because of the nuclear and mitochondrial genome standard used and the performed absolute quantitation with qPCR.

In our study, we found that mtDNA copy numbers were decreased in rat pancreas and small intestine tissues at all doses of RSG compared with the control. We determined the mtDNA copy numbers as unchanged in the liver and lymphocytes. Studies have founded that GPx and SOD enzyme activities are low in the pancreas and small intestine, and it has therefore been suggested that these cells may be sensitive to ROU (33,42,43). Illustrated that RSG causes the formation of ROU by increasing mitochondrial activity, on the other hand reducing excessive ROU by accelerating the expression of antioxidant enzymes (44,45). In this study, we thought that the decrease in mtDNA copy numbers in the pancreas and small intestine tissues of rats given RSG may be related to the low GPx and SOD enzyme activities in the pancreas and small intestine. Because of GPx and SOD enzyme activities are low in the small intestine and pancreas, RSG may not reduce ROU in these tissues or RSG may have an oxidant effect. The high GPx and SOD enzyme activities in the liver and the increase in the expression of these enzymes by RSG (33, 34) suggested that may be the reason for the decrease in $mtDNA^{4834}$ deletion frequency in liver tissue.

According to the results of our study, RSG shows different effects in tissues. Because of the decrease in $mtDNA^{4834}$ deletion frequency in liver tissue, made us think that RSG has no oxidant effect. However, the decrease in mitochondrial DNA copy numbers in the small intestine and pancreas tissues may be due to the oxidant effect of RSG. Further studies require to better explain this situation. For this purpose, may be planned to investigate the different effects of RSG in each tissue with cell culture studies, and also antioxidant enzymes can be tested at the tissue level.

References

1. Tack CJ, Smits P. Thiazolidinedione derivatives in type 2 diabetes mellitus. Neth J Med. 2006;64:166-74.
2. Spiegelman BM. PPAR-g adipogenic regulator and thiazolidinedione receptor. Diabetes. 1998; 47:507–14.

3. Da Ros R, Assaloni R, Ceriello A. The preventive anti-oxidant action of thiazolidinediones: a new therapeutic prospect in diabetes and insulin resistance. Diabet Med. 2004;21:1249-52.
4. Calkin AC, Forbes JM, Smith CM, Lassila M, Cooper ME, Jandeleit-Dahm KA et al. Rosiglitazone attenuates atherosclerosis in a model of insulin insufficiency independent of its metabolic effects. Aterioscler Thromb Vasc Biol. 2005;25:1903–9
5. Rong JX, Qiu Y, Hansen MK, Zhu L, Zhang V, Xie M et al. Adipose mitochondrial biogenesis is suppressed in db/db and high-fat diet-fed mice and improved by rosiglitazone. Diabetes. 2007;56:1751-60.
6. Brunmair B, Staniek K, Gras F, Scharf N, Althaym A, Clara R et al. Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? Diabetes. 2004;53:1052–9.
7. Scatena R, Bottoni P, Martorana GE, Ferrari F, De Sole P, Rossi C, et al. Mitochondrial respiratory chain dysfunction, a non-receptor-mediated effect of synthetic PPAR-ligands: biochemical and pharmacological implications. Biochem Biophys Res Comm 2004;319:967-73
8. García-Ruiz I, Rodríguez-Juan C, Díaz-Sanjuán T, Martínez MA, Muñoz-Yagüe T, Solís-Herruzol JA. Effects of Rosiglitazone on the Liver Histology and Mitochondrial Function in ob/ob Mice Hepatology. 2007;46:414-23.
9. Richter C. Reactive oxygen and DNA damage in mitochondria. Mutat. Res. 1992;275: 249–55.
10. Lenaz G. The Mitochondrial Production of ReactiveOxygen Species: Mechanisms and Implications in Human Pathology. IUBMB Life. 2001;52: 159–64
11. Sipos I, Tretter L, Adam-Vizi V. Quantitative relationship between inhibition of respiratory complexes and formation of reactive oxygen species in isolated nerve terminals. J. Neurochem. 2003; 84:112-8
12. Narayanan PK, Hart T, Elcock F, Zhang C, Hahn L, McFarland D et al. Troglitazone-induced intracellular oxidative stress in rat hepatoma cells: a flow cytometric assessment. Cytometry A. 2003;52:28–35.
13. Desjardins P, de Muys JM, Morais R. An established avian fibroblast cell line without mitochondrial DNA. Somat Cell Mol Genet. 1986;12:133-9.
14. Sawyer DE, Van Houten B. Repair of DNA damage in mitochondria. Mutat Res. 1999;434:161–76.
15. Cortopassi GA, Shibata DD, Soong NW, Arnheim N. A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. Proc Natl Acad Sci USA. 1992;89:7370–4.
16. Filburn CR, Edris W, Tamatani M, Hogue B, Kudryashova I, Hansford RG. Mitochondrial electron transport chain activities and DNA deletions in regions of the rat brain. Mech Ageing Dev. 1996;87:35–46.
17. Gadaleta MN, Rainaldi G, Lezza AM, Milella F, Fracasso F, Cantatore P. Mitochondrial DNA copy number and mitochondrial DNA deletion in adult

and senescent rats. Mutat Res. 1992; 275:181–93.

18. Edris W, Burgett B, Stine OC, Filburn CR. Detection and quantitation by competitive PCR of an age-associated increase in a 4.8kb deletion in rat mitochondrial DNA. Mutat Res. 1994; 316:69–78.
19. Nicklas JA, Brooks EM, Hunter TC, Single R, Branda RF. Development of a Quantitative PCR (TaqMan) Assay for Relative Mitochondrial DNA Copy Number and the Common Mitochondrial DNA Deletion in the Rat. Environmental and Molecular Mutagenesis. 2004;44:313–20.
20. Feinstein DL, Spagnolo A, Akar C, Weinberg G, Murphy P, Gavrilyuk V et al. Receptor-Independent Actions of PPAR Thiazolidinedione Agonists: Is Mitochondrial Function the Key? Biochem Pharmacol 2005; 70:177-88.
21. Bedir A, Aliyazicioğlu Y, Kahraman H, Yurdakul Z, Uysal M, Suvaci DE et al. Genotoxicity in rats treated with the antidiabetic agent, rosiglitazone. Environ Mol Mutagen. 2006;47:718-24.
22. Bedir A, Aliyazicioğlu Y, Bilgici B, Yurdakul Z, Uysal M, Suvaci DE et al. Assessment of genotoxicity in rats treated with the antidiabetic agent, pioglitazone. Environ Mol Mutagen. 2008;49:185-91.
23. Inmaculada GR, Rodriguez-Juan C, Diaz-Sanjuan T, Martinez MA, Munoz-Yague T and Solis-Herruzo J.A. Effects of Rosiglitazone on the Liver Histology and Mitochondrial Function in ob/ob Mice. Hepatology. 2007;46:414-23
24. Shiau CW, Yang CC, Kulp SK, Chen KF, Chen CS, Huang JW. Thiazolidenediones mediate apoptosis in prostate cancer cells in part through inhibition of Bcl-xL/Bcl-2 functions independently of PPARγ. Cancer Res. 2005;65:1561–9.
25. Ceolotto G, Gallo A, Papparella I, Franco L, Murphy E, Iori E et al. Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. Arterioscler Thromb Vasc Biol. 2007;27:2627-33.
26. Giannini S, Serio M, Galli A. Pleiotropic effects of thiazolidinediones: taking a look beyond antidiabetic activity. J Endocrinol Invest. 2004;27:982-91.
27. Girnun GD, Domann FE, Moore SA, Robbins ME. Identification of a functional peroxisome proliferator-activated receptor response element in the rat catalase promoter. Mol Endocrinol. 2002;16:2793-801.
28. Suematsu N, Tsutsui H, Wen J, Kang D, Ikeuchi M, Ide T et al. Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation. 2003;107:1418–23.
29. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci. 1994;91:10771-8.
30. Mandavilli BS, Santos JH, Van Houten B. Mitochondrial DNA repair and aging. Mutat Res. 2002;509:127-51.
31. Bravo-Nuevo A, Williams NK, Valter K, Stone J. Relationship between mitochondrial DNA damage andphotoreceptor death in developing and adult retina, assessed in normal and degenerative rat strains. Mitochondrion. 2007;7:340–6.

32. David LY, Bruce NA. Quantitation of age-related mitochondrial DNA deletions in rat tissues shows that their pattern of accumulation differs from that of humans. Gene. 1998;209:23–30.
33. Filser N, Margue C, and Richter C. Quantification of Wild-Type Mitochondrial DNA and Its 4.8-kb Deletion in Rat Organs. Biochemical and Biophysical Research Communications. 1997;233:102–7.
34. Garg R, Kumbkarni Y, Aljada A, Mohanty P, Ghanim H, Hamouda W. Troglitazone reduces reactive oxygen species generation by leukocytes and lipid peroxidation and improves flow-mediated vasodilatation in obese subjects. Hypertension. 2000;36:430–5.
35. Shay JW, Pierce DJ, Werbin H. Mitochondrial DNA copy number is proportional to total cell DNA under a variety of growth conditions. J. Biol. Chem. 1990;265:14802-7.
36. Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. Int J Biochem Cell Biol. 2005;37:822-34.
37. Bogacka I, Ukropcova B, McNeil M, Gimble JM, Smith SR. Structural and functional consequences of mitochondrial biogenesis in human adipocytes in vitro. J Clin Endocrinol Metab. 2005;90:6650-6.
38. Masuyama M, Iida R, Takatsuka H, Yasuda T, Matsuki T. Quantitative change in mitochondrial DNA content in various mouse tissues during aging. Biochimica et Biophysica Acta. 2005;1723:302–8.
39. Short KR, Bigelow ML, Kahl J. Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci. 2005;102:5618–23.
40. Menshikova EV, Ritov VB, Fairfull L, Ferrell RE, Kelley DE, Goodpaster BH. Effects of exercise on mitochondrial content function in aging human skeletal muscle. J Gerontol. 2006;61:534–40.
41. Lynn S, Borthwick GM, Charnley RM, Walker M, Turnbull DM. Heteroplasmic ratio of the A3243G mitochondrial DNA mutation in single pancreatic beta cells. Diabetologia. 2003;46:296–9. 93, 183, 212, 294, 315
42. Lenzen S, Drinkgern J, Tiedge M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. Free Radic. Biol. Med. 1996;20: 463–6.
43. Moghadasian MH, Godin DV. Gender-related regional antioxidant profiles in the gastrointestinal tract of the rat. Mol Cell Biochem. 1996;155:43–9.
44. Lin J, Handschin C, Spiegelman BM. Metabolic control through the PGC-1 family of transcription coactivators. Cell Metab. 2005;1:361–70
45. St. Pierre J, Lin J, Krauss S, Tarr PT, Yang R, Newgard CB et al. Bioenergetic analysis of peroxisome proliferator-activated receptör gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. J Biol Chem. 2003;278:26597–603.