Effect of Resveratrol on Proinflammatory Gene Expression in Pancreas of Streptozotocin Induced Diabetic Rats

Streptozotosin ile Diyabet Oluşturulmuş Sıçan Pankreasında Resveratrol Kullanımına Bağlı Proinflamatuar Gen İfadelenmelerinin Araştırılması

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ABSTRACT

Objective: Inflammation and oxidative stress play an important role in the development of Diabetes mellitus (DM). The production amount of increased reactive oxygen types in ß-cells that are sensitive to oxidative stress cause degeneration in insulin release and insulin resistance in Type 2 DM. Streptozotocin(STZ) breaks down ß-cells with the accumulation of free radicals by inhibiting pancreas superoxide dismutase. And Resveratrol (RSV) has an inhibitory effect on the degenaration of pancreatic ß cells. In this study, we aimed to examine the impact of expressions in NF-κB, TNFα, IL-6, iNOS and COX2 genes in STZ-induced diabetes in pancreases of rats on the complications of DM.

Methods: STZ-induced diabetes in pancreases of rats and RSV applied pancreatic tissues of rats after inducing diabetes are used. RNA isolation and cDNA synthesis are conducted for these tissues. mRNA expressions of NF-κB, TNFα, IL-6, iNOS and COX2 genes are realized with real-time PCR reaction and results are evaluated statistically.

Results: No significant difference is observed between control, sham control and control+DMSO groups on COX2, iNOS, NF-κB, TNFα and IL-6 in mRNA level. When control and diabetes groups are compared, it is observed that mRNA levels of COX2, iNOS, NF-κB, TNFα and IL-6 genes increased in mRNA expression levels of target genes in diabetes group. This presents a statistical meaning between two groups. And when diabetes and diabetes+RSV groups are compared, decrease in COX2, iNOS, NF-κB, TNFα and IL-6 genes are observed in mRNA levels. It is observed that this decrease is not statistically meaningful.

Conclusion: Data obtained shows that there is decrease in expression levels of inflammatory genes in diabetic rats and RSV application has no statistically significant effect on expression levels of these genes. From this point of view it can be said that only RSV application cannot change the expression levels of inflammatory genes associated with diabetes.

Key Words: Diabetes Mellitus, Streptozotocin, Resveratrol, Real-time PCR

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ÖZET

Amaç: İnflamasyon ve oksidatif stres, Diabetes mellitus (DM) gelişiminde önemli bir rol oynamaktadır. Oksidatif strese karşı duyarlı hücreler olan ß-hücrelerde artmış reaktif oksijen türlerinin üretim düzeyleri insülin salınımında bozulmalara, tip 2 DM'ye ve insülin direncine yol açmaktadır. Streptozotosin (STZ), pankreasta superoksit dismutazı inhibe ederek serbest radikallerin birikimiyle ß hücreleri yıkıma uğratmaktadır. Resveratrol (RSV) ise pankreatik ß hücrelerindeki bozulmayı inhibe edici bir etkiye sahiptir. Çalışmamızda, STZ ile diyabet oluşturulmuş sıçanlarda RSV'nin pankreasta NF-κB, TNFα, IL-6, iNOS ve COX2 genlerinin ifadelenmesinin DM'nin komplikasyonları üzerine etkisini araştırmayı amaçladık.

Yöntem: STZ ile diyabet oluşturulmuş ve diyabet oluşturulduktan sonra RSV uygulanmış sıçanlara ait pankreas dokuları kullanıldı. Bu dokulardan RNA izolasyonu ve cDNA sentezi yapıldı. NF- κB, TNFα, IL-6, iNOS ve COX2 genlerinin mRNA ifadelenme düzeyleri gerçek zamanlı PZR reaksiyonu ile gerçekleştirilerek sonuçlar istatistiksel olarak değerlendirildi.

Bulgular: Kontrol ile sham kontrol ve kontrol+DMSO grupları arasında COX2, iNOS, NF-κB, TNFα ve IL-6 mRNA düzeyinde anlamlı bir fark gözlenmedi. Kontrol ile diyabet grubu karşılaştırıldığında COX2, iNOS, NF-κB, TNFα ve IL-6 genlerinin mRNA düzeylerinde, diyabet grubunda hedef genlerin mRNA ifadelenme düzeylerinde artış olduğu gözlendi ve bu iki grup arasında istatistiksel olarak anlam bulundu. Diyabet ile diyabet+RSV grubu karşılaştırıldığında ise COX2, iNOS, NF-κB, TNFα ve IL-6 genlerinin mRNA düzeylerinde bir azalma gözlenirken, bu azalışın istatistiksel açıdan anlamlı olmadığı gözlendi.

Sonuç: Elde ettiğimiz veriler, diyabetik sıçanlarda inflamatuar genlerin ifade düzeylerinde artış olduğunu ve RSV uygulamasının bu genlerin ifade düzeyleri üzerinde istatistiksel olarak anlamlı bir etkisinin olmadığını göstermektedir. Buradan hareketle sadece RSV uygulamasının diyabetle ilişkili inflamatuar genlerin ifade düzeylerini değiştiremeyeceği söylenebilir.

Anahtar Sözcükler: Diabetes Mellitus, Streptozotosin, Resveratrol, Gerçek zamanlı PZR

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INRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease resulting from absence or insufficiency of insulin production, which triggers hyperglycemia and characterized by metabolic disorders (1). Hyperglycemia leads to Polydipsia, poliphagia, polyuria, weight loss, blurred vision. Insulin is a hormone which enables glucose to enter the cell, produced in pancreas and regulates plasma glucose (2). Cell cannot convert glucose to energy in insufficiency, absence or incorrect production of insulin, thereby plasma glucose level increases and results in hyperglycemia (3). Type I DM results from auto-immune destruction of beta cells in Langerhans islets of pancreas, type II DM results from insulin resistance developing due to obesity and insufficient physical activity in middle and advanced age (4).

Streptozotocin (STZ) inhibits superoxide dismutase which is a free radical scavenger in pancreas and beta cells are destructed due to free radical accumulation (5). Type I DM is usually induced with chemical agents STZ and alloxan (ALX) in experimental animals. STZ and ALX are defined as diabetogenic agents due to beta cell toxic properties. Three phase effect induced by these agents includes plasma glucose elevation due to abrupt liver glycogen degradation within two hours (phase I), hypoglycemia (phase II), permanent hypoglycemia (phase III) (6).

Resveratrol (RSV) which belongs to polyphenol phytoalexin class is a stillbenoid which is a by-product of stillben obtained from herbs with stillben synthase enzyme (7). RSV has an anti-bacterial, anti-fungal and anti-oxidant effect. It also increases insulin sensitivity, reduces plasma glucose level and increases mitochondrial capacity. Therefore RSV is useful for protection from DM and relieving DM complications. Studies have revealed that RSV improves metabolic values, reduces plasma glucose and triglyceride concentration, effects of insulinemia (8).

In the present study, we aimed to investigate the influences of NF-κB, TNFα, IL-6, iNOS and COX2 gene expressions in pancreas on DM complications in rats with STZ-induced DM.

MATERIAL and METHODS

Groups

Six groups were used in the study. First is control group (n=6). Second is sham (sodium citrate) group (n=6), third is DM group (n=6), fourth is control + DMSO group (n=6), fifth is sodium citrate + RSV group (n=6), sixth is DM + RSV group (n=6). Pancreatic tissues were washed with sterile saline in sterile plates and divided to small parts (50-100 mg) using sterile knives in accordance with RNA isolation rules. Tissue samples which were taken to 1.5 ml of DNas and RNas-free 1.5 ml steril tube, immediately frozen in liquid nitrogen and stores at -80 °C until the time of study.

RNA extraction and reverse transcription

Total RNA was extracted from pancreatic tissue (approximately 50-100 mg) using RNA isolation kit (peq GOLD TriFast Tissue isolation kit, Peqlab, Erlangen, Germany). RNA of each sample were determined by measuring the absorbance at 260 nm using the Nanodrop spectrophotometer (NanoDrop ND-1000, Montchanin, DE, USA) and µl/ µg values were calculated. Total RNA (1 µg) was reverse transcribed in a 20-µL reaction mixture using random hexamers and Transcriptor First-Strand cDNA Synthesis kit (Transcriptor first strand cDNA synthesis kit, Roche, Germany) according to manufacturer instructions.

Real-time PCR (RT-PCR) analysis

NF-κB1, COX2, iNOS, TNFα and IL-6 mRNA expression levels were measured using RT-PCR with the LightCycler instrument. β-actin (ACTB) was used as a housekeeping gene in order to normalize NF-κB1, COX2, iNOS, TNFα and IL-6 expression levels. Amplifications were performed using cDNA, gene specific primers, Universal Probe Library (UPL) probe and LightCycler 480 Probe mix (Roche, Germany) according to manufacturer instructions.

Probes and primers spanning exon-exon boundaries for each gene assay were designed using the UPL Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000). The primer and UPL probe numbers are presented in Table 1. Elevation or reduction in NF-κB1, COX2, iNOS, TNFα and IL-6 mRNA levels in test groups were determined using Cp (Crossing point) values with Pfaffl method (9).

Tablo 1. Gene specific primer sequences and probe numbers.

Gene	Forward primer	Reverse primer	UPL probe No.
ACTB	5'CCCGCGAGTACAACCTTCT3'	5'CGTCATCCATGGCGAACT3'	17
NF-κB	5'ACTGCTCAGGCCCACTTG3'	5'TGTCATTATCTCGGAGCTCATCT3'	25
COX2	5'ACCAACGCTGCCACAACT3'	5'GGTTGGAACAGCAAGGATTT3'	77
NOS2	5'ACCATGGAGCATCCCAAGT3'	5'CAGCGCATACCACTTCAGC3'	128
TNFα	5'TGAACTTCGGGGTGATCG3'	5'GGGCTTGTCACTCGAGTTTT3'	63
IL6	5'CCCTTCAGGAACAGCTATGAA3'	5'ACAACATCAGTCCCAAGAAGG3'	20

Statistical Analyses

Data of NF-κB1, COX2, iNOS, TNFα and IL-6 mRNA expressions in pancreatic tissues of rats in control and DM groups were analyzed by the pairwise fixed reallocation randomization test as statistical model included in the REST© software (Relative Expression Software Tool, 2005 Beta V1.9.9) developed for group-wise comparison and statistical analysis of relative expression results. P values lower than 0.05 were considered to be significant. Diabetes-related weight and glucose alterations of rats were compared using non-parametric Kruskal-Wallis test and the Mann-Whitney U-test. The data were evaluated using the SPSS statistical software (version 22.0 for Windows; SPSS Inc., Chicago, IL, USA).

RESULTS

Pancreatic tissues of 36 Wistar albino rats weighing 250-300 mg were used and diabetes was created using STZ. Rats whose fasting plasma glucose was above 250 mg/dl were accepted as diabetic (10).

When control and sham control, control+ DMSO groups were compared, a statistically significant difference was not detected between groups with regard to mRNA expressions of COX2, iNOS, NF-κB1, TNFα and IL-6 genes ($p>0.05$). Similarly, when sham control and sham control + RSV groups were compared, a statistically significant difference was not detected between groups with regard to mRNA expressions of COX2, iNOS, NF-κB1, TNFα and IL-6 genes ($p>0.05$). When control and DM group were compared, a statistically significant increase was detected in expression levels of COX2, iNOS, NF-κB1, TNFα and IL-6 in DM group ($p<0.05$). When DM and DM+ RSV groups were compared, while a significant decrease was detected in mRNA levels of COX2, iNOS, NF-κB1, TNFα and IL-6 genes, this decrease was not found statistically significant ($p>0.05$). mRNA alterations of COX2, iNOS, NF-κB1, TNFα and IL-6 between groups are shown in Figure 1.

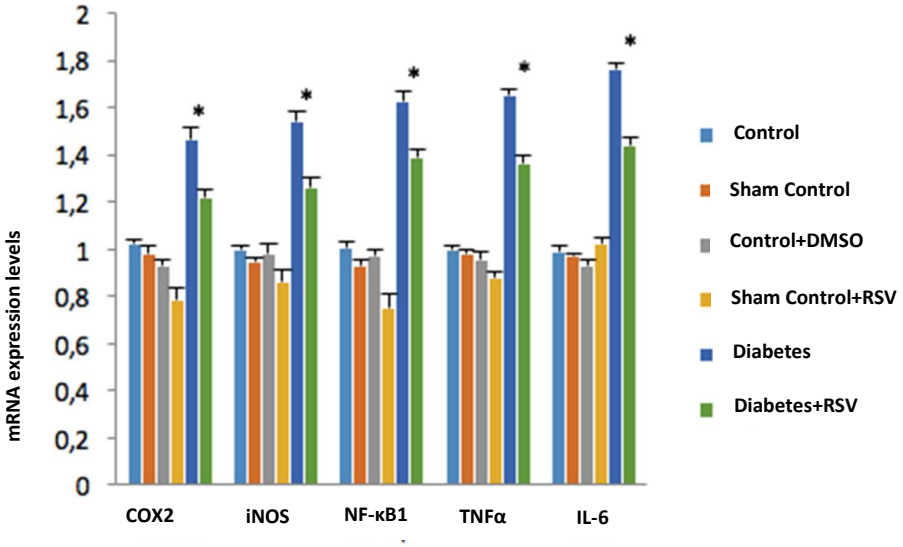

Figure 1. mRNA alterations of COX2, iNOS, NF-κB1, TNFα and IL-6 between groups.
Expression levels of target genes are normalized based on beta-actin mRNA expression level *; p<0.05.

DISCUSSION

DM is among the diseases with gradually increasing prevalence in our country and worldwide. Number of diabetic patients was 175 million worldwide in 2000 and it is estimated to be 300 million in 2025 according to WHO data. In Turkey, diabetes prevalence was reported as 7.2% in TURDEP-I and elevated to 13.7% with 90% increase in TURDEP-II conducted in 2010 (11).

The presented study aimed at investigating mRNA expressions of inflammatory NF-κB1, COX2, iNOS, TNFα and IL-6 genes in pancreas. When control and diabetes groups were compared, mRNA expressions of NF-κB1, TNFα and IL-6 genes were detected to significantly increase in pancreatic tissues. Besides, iNOS and mRNA levels were also detected to significantly increase. Saha and Ghosh have reported a statistically significant increase in inflammatory cytokines like TNFα and IL-6, and hepatic NF-κB expression levels in rats with STZ-induced DM (12). Lekshmi et al. detected that mRNA expressions of TNFα, IL-6 and NF-κB significantly increased in rats with STZ-induced DM (13). Akcılar et al. detected that TNFα, IL-6 and iNOS expression levels significantly increased in pancreatic tissues of rats with STZ-induced DM (14). He et al. detected that iNOS expression levels significantly increased in pancreatic tissues of rats with STZ-induced DM compared to control group (15). Results of our study are similar with those of the abovementioned studies.

No adverse effects of RSV were detected in the studies. Williams et al. have investigated 50 mg/kg, 150 mg/kg, 500 mg/kg doses in three rat groups and elevated the dose up to 700 mg/kg however they have detected no adverse effects (16). Horn et al. have investigated the effects of over dose RSV administration. No direct fatal effect was observed in even 1000 mg/kg dose (17). No alteration was observed after RSV administration in the studies conducted with low doses (18).

The studies investigating the positive effects of RSV indicated that RSV dose is similar. Lai et al. have investigated NF-κB activation with RSV, administered 15 mg/kg and 30 mg/kg RSV and revealed that NF-κB showed a dose-dependent increase (19). The dose of 10 mg/kg/day administered in our study is supported by literature data (10).

The study of Crofford has indicated that COX enzymes, particularly COX2 is an important factor in inflammatory diseases like DM (20). Martinez and Morreno have also revealed the inflammatory effect of COX2 (7). Yar et al. have detected that RSV did not have a significant effect on COX gene and protein expression (21).

Newton et al. have revealed that mRNA of COX2 is inhibited through suppression of AP-1 and NF-κB (22). Zamon et al. have also indicated the influence of NF-κB on iNOS transciption and influence of iNOS transcription on inflammation (23). In our study, a significant decrease was detected in COX2, iNOS, NF-κB1 mRNA levels in pancreas of the rats with STZ-induced DM however a significant difference was not detected.

Bertelli et al. have revealed that RSV showed anti-inflammatory effect through inhibiting TNFα-related endothelial cell activation based on the association between iNOS expression and TNFα (24). Csiszar et al. have detected that RSV suppressed TNFα-related NF-κB activation and showed anti-inflammatory effect (25). In our study, a significant reduction was detected in TNFα mRNA levels in pancreas of the rats which were administered RSV and had STZ-induced DM however a significant difference was not detected.

Zhong et al. detected the influence of RSV on IL-6 release and also one of the molecules which were planned to be inhibited could be suppressed with RSV (26). In our study, a reduction was detected in IL-6 and mRNA levels in pancreas of the arts which were administered RSV however the difference was not found statistically significant.

Gao et al. have studied the hearts of STZ-adminsitered rats and revealed that RSV decreased diabetes-induced cardiac dysfunction through inhibiting inflammatory factors like TNFα, IL-6 and IL-1β (27). Zheng et al. have found out that RSV led to a reduction in nuclear translocation in NF-κB and in TNFα expression in vascular wall and TNFα, IL-1β, IL-6 levels (28). Kumar and Sharma revealed that RSV administration reduced p65 expression and improved high COX2, TNFα, IL-6 levels (29). Pektaş etal. have investigated the influence of RSV in liver in their study conducted with male Wistar rats as in our study and found TNFα, IL-6, iNOS levels significantly higher in diabetic rats compared to control group. The authors have revealed a significant reduction in rats which were administered RSV compared to the ones which were not administered RSV. In our study, a reduction was detected in COX2, iNOS, NF-κB1, TNFα and IL-6 mRNA when diabetic rats group and the diabetic rat group which were administered RSV were compared however a significant difference was not detected.

CONCLUSION

An increase was detected in oxidative stress and pro-inflammatory gene expressions in diabetic rats and RSV administration was detected to lead to a reduction in expression of these genes however a statistically significant difference was not found. The results of the present study have indicated that RSV did not have a significant effect on oxidative stress and inflammatory genes.

Conflict of interest

No conflict of interest was declared by the authors.

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