

# Oestradiol Treatment Counteracts the Effect of Fructose-Rich Diet on Matrix Metalloproteinase 9 Expression and NFκB Activation

(fructose-rich diet / matrix metalloproteinase 9 / NFκB / oestradiol / heart / rat)

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**Abstract.** Fructose-rich diet induces metabolic changes similar to those observed in metabolic syndrome. Among other matrix metalloproteinases, MMP-9 has an important role in adverse cardiac remodelling and might have a role in the development of cardiovascular disorders associated with metabolic syndrome. The changes of MMP-9 expression could be mediated via the NFκB pathway. In this study we investigated the effect of fructose-rich diet on MMP-9 expression in the heart of male and female rats, along with the effect of fructose-rich diet and oestradiol on MMP-9 expression in ovariectomized females. We further assessed the effect of fructose-rich diet and oestradiol on NFκB activation, measured as the level of p65 phosphorylation at Ser 276. The results showed that the diet regime did not affect the heart mass. Higher MMP-9 gene expression was found in cardiac tissue of male rats fed the fructose-rich diet than in females on the same diet regime. In ovariectomized

females, fructose-rich diet upregulated MMP-9 protein and mRNA expression in the heart, as well as phosphorylation of the p65 subunit of NFκB at Ser 276. Oestradiol replacement therapy reverted these changes in the heart of ovariectomized females. This study has shown that oestradiol could revert the early molecular changes in MMP-9 expression induced by fructose-rich diet that occurred before cardiac hypertrophy development by decreasing phosphorylation of the NFκB p65 subunit at Ser 276.

## Introduction

Metabolic syndrome is described as a pro-inflammatory condition associated with increased risk for diabetes, accelerated atherosclerosis and increased incidence of cardiovascular diseases (Isomaa et al., 2001; Grundy et al., 2004; Paoletti et al., 2006). It combines several risk factors for cardiovascular disease occurrence: hypertension, insulin resistance, hyperinsulinaemia, dyslipidaemia and abdominal obesity (Eckel et al., 2005; Grundy, 2007). Numerous experiments on animals and human demonstrated that fructose-rich diet (FRD) might induce metabolic changes similar to those observed in metabolic syndrome (Dai and McNeil, 1995; Sharabi et al., 2007; Orron-Herman et al., 2008; Axelsen et al., 2010; Dekker et al., 2010; Tappy and Le, 2010).

Recently, it was suggested that matrix metalloproteinases (MMPs) might have a role in the development of cardiovascular disorders associated with metabolic syndrome (Cicero et al., 2007; Miksztowich et al., 2008). MMPs are calcium-dependent, zinc-containing endopeptidases, which are involved in extracellular matrix (ECM) remodelling (Bode and Maskos, 2003; Visse and Nagase, 2003). Knowing that adverse ECM remodelling of the myocardium and vasculature leads to the development of cardiovascular disorders, it is not surprising that MMPs represent an important biological components in the myocardium (Spinale, 2002; Lopes et al., 2004; Newby, 2005).

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Abbreviations: ACE – angiotensin-converting enzyme, AT1R – angiotensin type 1 receptor, BCA – bichinchonic acid, cDNA – complementary DNA, E2 – 17β-oestradiol, ECM – extracellular matrix, FRD – fructose-rich diet, IκB – inhibitor κB, IL – interleukin, MMP – matrix metalloproteinase, ND – normal diet, NFκB – nuclear factor κ-light-chain-enhancer of activated B cells, OVX – ovariectomized, PVDF – polyvinylidene fluoride, Q-PCR – quantitative real-time PCR, rRNA – ribosomal RNA, SDS – sodium dodecyl sulphate, SHHF – spontaneously hypertensive heart failure, TNF-α – tumour necrosis factor α.

MMP-9 or gelatinase B, which is an inducible enzyme tearing down type IV collagen (Gioia et al., 2009), has been shown to play an important role in the pathogenesis of a wide spectrum of cardiovascular disorders (Dollery et al., 1995; Lindsay et al., 2002). An elevated level of MMP-9 represents one of the risk factors for development of cardiovascular diseases and myocardial infarction (Ferroni et al., 2003). An increasing circulating MMP-9 level and activity was found in patients with metabolic syndrome (Cicero et al., 2007; Goncalves et al., 2009), hyperglycaemia (Lee et al., 2005), and type 2 diabetes mellitus (Uemura et al., 2001). Previous studies have shown that FRD enhanced MMP-9 activity in rat smooth muscle cells (Lu et al., 2013), as well as the MMP-9 protein level in aortic tissue of ApoE-KO mice (Cannizzo et al., 2012). As far as we know, there are no experimental studies investigating the effects of FRD on MMP-9 expression in the heart. Because MMP-9 expression shows gender differences with higher levels detected in male rats (Woodrum et al., 2005), we considered interesting to examine whether FRD affects MMP-9 expression in a gender-specific manner and to evaluate the influence of female sex hormones on MMP-9 expression in the context of fructose-rich diet.

The changes of MMP-9 expression could be mediated via the nuclear factor  $\kappa$ -light-chain-enhancer of activated B cells (NF $\kappa$ B) pathway (Guo et al., 2008). NF $\kappa$ B is activated through phosphorylation of the p65 subunit at Ser 276 (Kim et al., 2012). Therefore, we hypothesized that phosphorylation of the p65 subunit at Ser 276 could be implicated in the induction of MMP-9 expression.

The purpose of this study was to investigate the effect of FRD on MMP-9 expression in the heart of male and female rats and to evaluate the effect of oestrogen on MMP-9 expression in fructose-fed rats. We further assessed the effect of FRD and 17 $\beta$ -oestradiol (E2) on NF $\kappa$ B activation, measured as the level of p65 phosphorylation at Ser 276.

## Material and Methods

### Chemicals

We purchased fructose from API-PEK (Becej, Serbia). Anti-NF $\kappa$ B p65 (phospho S276) and anti-MMP-9 antibodies were obtained from Abcam (Cambridge, UK). Secondary anti-rabbit antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA). 17 $\beta$ -oestradiol (E2) was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

### Animals

This research was approved by the official Vinca Institute's Ethical Committee for Experimental Animals and conformed to the Directive 2010/63/EU of the European Parliament. Twenty-one-day-old male and female Wistar rats were separated from their mothers. Rats were bred at the Animal Facility of Vinca Institute of Nuclear Sciences. Male rats were divided into a nor-

mal diet (ND-male) and a fructose-fed group (FRD-male). Females were divided into an intact and an ovariectomized group. Intact females were further divided into a normal diet (ND-female) and a fructose-rich diet (FRD-female) group. Ovariectomized females were divided into a normal diet group (ND-OVX), a fructose-rich diet group (FRD-OVX) and animals fed the fructose-rich diet and subjected to oestradiol replacement therapy (FRD-OVX+E2). Animals held on normal diet had free access to tap water and normal laboratory chow. Animals fed the fructose-rich diet also had standard laboratory chow, while 10% (w/v) fructose solution replaced the tap water. This diet regime lasted for nine weeks. In the ovariectomized group, ovariectomy was performed two weeks before sacrifice under ketamine (40 mg/kg, intraperitoneally) – xylazine (5 mg/kg, intraperitoneally) anaesthesia. E2 replacement therapy (40  $\mu$ g/kg, subcutaneously, every second day to achieve concentration near the physiological level) started a day after ovariectomy and continued until the day before sacrifice (Sales et al., 2010). This duration of E2 replacement therapy was shown to allow E2 to achieve long-term genomic effects, but is also sufficient for E2 to influence non-genomic signalling mechanisms (Koricnac et al., 2009). In order to avoid the effects of injection stress, ND-OVX and FRD-OVX animals were injected with vehicle (linseed oil). Each experimental group contained nine animals (N = 9), which means that a total of 63 animals participated in the experiment.

### Measurement of body mass and mass of heart

Body mass was measured at the end of the experiment. Heart mass was determined immediately after killing the animals. The hearts were excised, washed in saline and dried before the measurement.

### Tissue homogenization and Western blot

Heart tissue was homogenized with an Ultra-turrax homogenizer in homogenization buffer (pH 7.4; containing: 10 mM Tris, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, protease inhibitors – 2 mM PMSF, 10  $\mu$ g/ml leupeptin, and 10  $\mu$ g/ml aprotinin and phosphatase inhibitors – 1 mM Na<sub>3</sub>VO<sub>4</sub> and 10 mM NaF) and homogenates were centrifuged at 600 g for 20 min. The supernatants were further ultracentrifuged at 100,000 g for 60 min. To determine the protein concentration we used the BCA method. After boiling in Laemmli sample buffer, supernatants were used for Western blot analysis. Equal amounts of proteins (100  $\mu$ g/lane) were fractioned by 10% SDS polyacrylamide gels and transferred onto PVDF membranes. Membranes were blocked with 5% bovine serum albumin for 2 h at room temperature, after which they were incubated with primary antibodies MMP-9 (ab7299, dilution 1 : 500) and NF $\kappa$ B p65 (Phospho S276) (ab30632, dilution 1 : 1000) overnight at 4 °C. Membranes were subsequently washed and incubated with peroxidase-labelled secondary anti-rabbit antibody for two hours at room temperature. Signals were detected with ECL reagents.

All Western blot experiments were performed in triplicates. To assess the equal protein loading, we used  $\beta$ -actin as a loading control. We optimized the protein quantity, primary and secondary antibody concentrations, and conditions of signal development in order to avoid ECL signal saturation. Films were scanned and intensities were determined using ImageJ software (NIH, Bethesda, MD).

### RNA isolation and expression by real-time polymerase chain reaction

Total RNA was extracted from the heart tissue using Trizol reagent (Ambion Inc., Austin, TX) according to the protocol recommended by the manufacturer. The quantity of RNA was assessed spectrophotometrically (NanoDrop® ND-1000, Thermo Scientific, Rockford, IL). In order to eliminate the possible contamination with genomic DNA, purified RNA was treated with DNase I. First strand cDNAs were generated from 2  $\mu$ g of total pure RNA and First Strand cDNA Synthesis kit, with oligodT18 primers, according to manufacturer's instructions (Fermentas, Vilnius, Lithuania). Quantitative real-time PCR (Q-PCR) amplification was performed in duplicate using the ABI Real-time 7500 system (ABI, Foster City, CA). The relative levels of specific MMP-9 mRNA in the heart were assessed by amplification in a total volume of 25  $\mu$ l by pre-developed TaqMan® Gene Expression Assays Rn00579162\_m1. 18s rRNA was used as an internal reference (Gene Expression Assays ID Hs99999901\_s1). Cycling parameters were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of a denaturation step at 95 °C for 15 s, and an annealing step at 60 °C for 60 s. In order to process the data and compare the differences in relative gene expression between groups we used the  $2^{-\Delta Ct}$  method. The calculated  $C_t$  values for MMP-9 in response to various treatments were normalized to the respective  $C_t$  values for 18S rRNA.

### Statistical analysis

Data were analysed using the GraphPad Prism 5 statistical package (San Diego, CA). All data are expressed as mean  $\pm$  standard deviation, for nine animals per each experimental group. Values of continuous variables with skewed distribution were compared by the nonparametric Mann-Whitney U test, which was used to examine the statistical significance among experimental groups.

The differences in relative gene expression between groups were calculated using  $2^{-\Delta Ct}$  methods (Livak and Schmittgen, 2001). Values of  $P < 0.05$  were considered statistically significant.

## Results

Body mass between female and male rats was significantly different in both, ND and FRD group ( $P < 0.001$ , ND Female vs. ND Male;  $P < 0.05$ , FRD Female vs. FRD Male) (Table 1). In males, FRD did not alter the body mass, while in females FRD increased the body mass ( $P < 0.05$ , ND Female vs. FRD Female). The mass of the heart was not affected by the diet regime regardless of sex. Males that were fed normal diet had significantly higher mass of the heart than females on the same diet regime ( $P < 0.01$ , ND Female vs. ND Male). The heart-to-body ratio did not differ among the groups.

To examine the effect of FRD on *MMP9* gene expression we performed quantitative RT-PCR analysis. The results showed higher *MMP9* gene expression in FRD-male compared to the FRD-female group ( $P < 0.01$ , FRD Female vs. FRD Male) (Fig. 1a). FRD did not change *MMP9* gene expression in females. In males, FRD up-regulated *MMP9* expression, but this showed not to be statistically significant. The fact that the *MMP9* gene showed higher expression in FRD-males compared to FRD-females made us to focus on the role of oestradiol in the regulation of *MMP9* expression in the heart of fructose-fed rats.

We therefore compared the effects of FRD and subsequent oestradiol treatment on ovariectomized female rats. In ovariectomized females, FRD upregulated *MMP9* gene expression ( $P < 0.01$ , ND-OVX vs. FRD-OVX). In contrast, E2 replacement therapy significantly down-regulated *MMP9* gene expression in fructose-fed ovariectomized animals ( $P < 0.01$ , FRD-OVX vs. FRD-OVX+E2) and decreased it even below the level detected in ND ovariectomized females ( $P < 0.01$ , ND-OVX vs. FRD-OVX) (Fig. 1b).

As presented in Table 2, neither FRD nor E2 replacement therapy affected body mass or the mass of the heart. This showed that heart hypertrophy had not yet occurred.

To (semi)quantitatively assay the MMP-9 protein level in the context of FRD and subsequent E2 replacement therapy, we performed Western blot analysis. These results are presented in Fig. 2. Similarly as for the mRNA level, FRD significantly increased the MMP-9

Table 1. Body mass and the mass of the heart of intact females and males

	ND Female	FRD Female	ND Male	FRD Male
Body mass (g)	263.20 $\pm$ 30.26	279.50 $\pm$ 20.52*	338.90 $\pm$ 40.45 <sup>#</sup>	311.30 $\pm$ 37.58 <sup>§</sup>
Mass of the heart (g)	0.83 $\pm$ 0.13	0.83 $\pm$ 0.08	1.05 $\pm$ 0.14**	0.94 $\pm$ 0.16
Heart to body ratio ( $\times 10^{-3}$ )	3.17 $\pm$ 0.16	3.04 $\pm$ 0.21	3.11 $\pm$ 0.19	3.00 $\pm$ 0.26

Values are expressed as means  $\pm$  standard deviations for a total of 9 rats in each experimental group. Mann-Whitney U test was used to compare the values of skewed continuous variables between groups; in all tests, P values  $< 0.05$  were considered statistically significant. ND – normal diet, FRD – fructose-rich diet. \* $P < 0.05$  ND Female vs. FRD Female, <sup>#</sup> $P < 0.001$  ND Female vs. ND Male, <sup>§</sup> $P < 0.05$  FRD Female vs. FRD Male, \*\* $P < 0.01$  ND Male vs. ND Female.

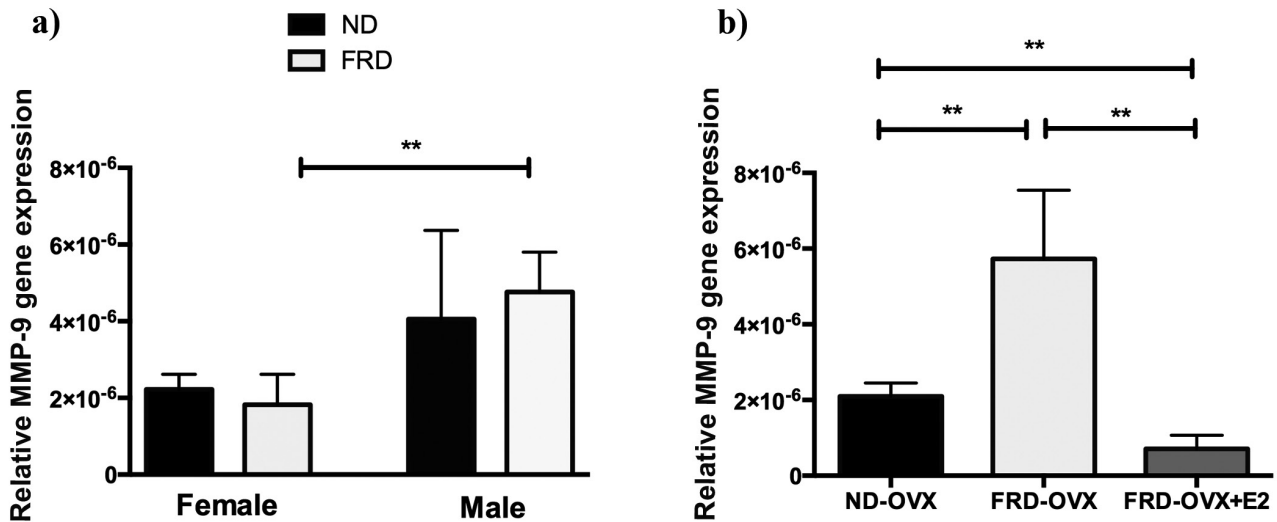


Fig. 1. Relative *MMP9* gene expression.

The cDNAs from heart tissue specimens were used as templates in RT-qPCR for relative quantification of MMP-9 mRNA expression. For each specimen, the expression level of MMP-9 mRNA was normalized to the housekeeping gene 18S rRNA. Relative expression of MMP-9, normalized against the housekeeping gene, was calculated using the comparative  $C_t$  method. (a) Relative *MMP9* gene expression in male and female rats fed a standard laboratory food and fructose-rich diet. Males on fructose-rich diet showed higher *MMP9* gene expression than females on the same diet. ND – normal diet, FRD – fructose-rich diet, \*\*  $P < 0.01$ . (b) Relative *MMP9* gene expression in ovariectomized fructose-fed rats with or without oestradiol replacement. OVX – ovariectomy, E2 – oestradiol treatment, \*\*  $P < 0.01$ .

protein level in the heart of ovariectomized rats ( $P < 0.001$ , ND-OVX vs. FRD-OVX). In contrast, oestradiol treatment significantly decreased MMP-9 protein expression compared to both, FRD-OVX ( $P < 0.01$ , FRD-OVX vs. FRD-OVX+E2) and ND-OVX animals ( $P < 0.001$ , ND-OVX vs. FRD-OVX+E2).

Having in mind that NF $\kappa$ B regulates MMP-9 expression, we investigated the effect of FRD and E2 (in the context of FRD) on activation of this transcription factor. As it was presented in Fig. 3, FRD increased phosphorylation of the p65 subunit at Ser 276 ( $P < 0.01$ , ND-OVX vs. FRD-OVX), thereby activating NF $\kappa$ B, while E2 replacement therapy reduced p65 phosphorylation ( $P < 0.05$ , FRD-OVX vs. FRD-OVX+E2) and decreased it to the level below that detected in ND-OVX females ( $P < 0.01$ , ND-OVX vs. FRD-OVX+E2).

## Discussion

The major finding of this study is that FRD increases MMP-9 expression in ovariectomized female rats even

before cardiac structural changes have been detected. This change in MMP-9 expression is oestrogen dependent and is in line with increased phosphorylation of the p65 subunit of NF $\kappa$ B transcription factor, which occurs during the FRD regime.

The results showed that 9-week treatment with 10% fructose solution did not cause any changes in the mass of the heart. This indicated that heart hypertrophy had not yet developed. Other studies demonstrated that either increased concentration of the consumed fructose or prolonged period of the diet regime leads to development of cardiac hypertrophy, excessive collagen deposition and increased stiffness of the left ventricle (Kobayashi et al., 1993; Kamide et al., 2002; Patel et al., 2009).

Previously, we have reported that 10% fructose-rich diet regime in the duration of 9 weeks leads to development of cardiac insulin resistance (Zakula et al., 2011; Romić et al., 2013). We also reported that FRD significantly elevated blood pressure in male rats, while in females we did not observe any differences in blood pressure as a consequence of FRD (Koricanac et al., 2013).

Table 2. Body mass and the mass of the heart of ovariectomized rats

	ND-OVX	FRD-OVX	FRD-OVX+E2
Body mass (g)	293.20 $\pm$ 23.09	286.70 $\pm$ 23.87	280.60 $\pm$ 18.15
Mass of the heart (g)	0.85 $\pm$ 0.08	0.84 $\pm$ 0.10	0.84 $\pm$ 0.07
Heart to body ratio ( $\times 10^{-3}$ )	2.92 $\pm$ 0.13	3.00 $\pm$ 0.14	2.98 $\pm$ 0.15

Data presented are means  $\pm$  standard deviations for 9 rats per each experimental group. Mann-Whitney U test was used to compare the values of skewed continuous variables between groups; in all tests,  $P$  values  $< 0.05$  were considered statistically significant. None of the comparisons showed statistical significance. FRD and E2 replacement therapy did not affect body mass and the mass of the heart. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomized, E2 – oestradiol treatment.

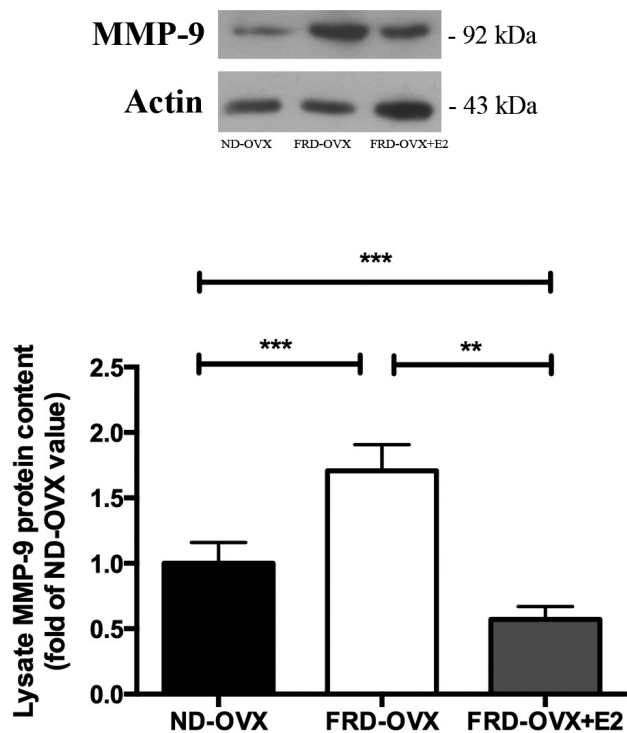


Fig. 2. MMP-9 protein expression in ovariectomized fructose-fed rats, rats with or without oestradiol treatment. Fructose-rich diet increased MMP-9 protein expression, while oestradiol replacement therapy reverted this change and decreased MMP-9 expression even below the level detected in animals on normal diet. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomy, E2 – oestradiol treatment, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Adverse ECM remodelling in the myocardium finally results in mortality and morbidity associated with various cardiovascular diseases including hypertension, myocardial infarction, cardiomyopathy, and finally heart failure (Cohn et al., 2000). MMPs are proposed to lead toward LV dilatation and heart failure. The MMP-9 protein level and activity were significantly elevated in the spontaneously hypertensive heart failure (SHHF) rat model (Li et al., 2000). The results of other studies also indicate that MMP-9 has an important role in the cardiac remodelling associated with hypertension (Tayebjee et al., 2004; Chiao et al., 2012). MMP-9 directly promotes myofibrillar transformation (Jiang et al., 2013), which is important in the development of cardiac fibrosis and scar formation (Porter and Turner, 2009).

In the presented study we detected elevated *MMP9* gene expression in FRD-male rats compared to FRD-females. Further, we found that FRD upregulated *MMP9* gene expression in ovariectomized females. A previous study showed increased MMP-9 activity in intimal smooth muscle cells of fructose-fed rats (Lu et al., 2013). Because E2 could reduce MMP-9 expression (Vegeto et al., 2001), we have further investigated whether E2 could revert changes in *MMP9* gene expression induced by FRD. Indeed, we showed that E2 replacement was able to revert these changes. This confirms that E2 is

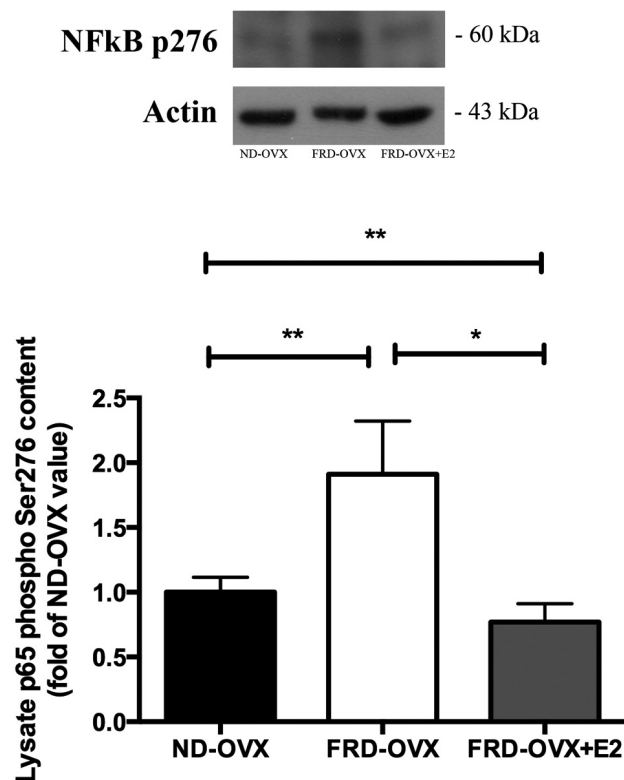


Fig. 3. Effects of fructose-rich diet and oestradiol on phosphorylation of NFκB p65 subunit at Ser 276. Fructose-rich diet increased phosphorylation of p65 subunit at Ser 276, thereby activating NFκB, while E2 replacement therapy reduced p65 phosphorylation. ND – normal diet, FRD – fructose-rich diet, OVX – ovariectomy, E2 – oestradiol treatment, \* $P < 0.05$ , \*\* $P < 0.01$ .

able to protect rats from changes in the *MMP9* gene expression that are consequences of FRD. As a further confirmation of this protective effect of E2 in the context of FRD, beside results obtained at the gene level, we have also studied the MMP-9 changes at the protein level. Changes in the MMP-9 protein level were in the same direction as those observed for gene expression, except that E2 decreased the MMP-9 protein level even below the level detected in ND-OVX animals. MMP-9 is a key contributor to adverse myocardial remodelling. Because the cellular source of MMP-9 appears to be predominantly infiltrating inflammatory cells (Mukherjee et al., 2006), it is not surprising that inhibition of MMP-9 expression or activity reduced myocardial inflammation and remodelling (Spinale, 2007). Based on the aforementioned findings, we can conclude that FRD causes changes in MMP-9 expression in the rat heart even before structural changes have occurred, and that these early molecular changes are reversible with E2. This suggests that E2 could protect the heart from possible harmful effects of FRD.

Angiotensin II was shown to promote cardiovascular remodelling through the increase of MMP-9 expression via AT1R, while telmisartan (AT1R blocker) and captopril (angiotensin-converting enzyme inhibitor) suppress MMP-9 expression and activities (Okada et al., 2008,

2009, 2010). Several studies have confirmed that NF $\kappa$ B is absolutely required and the most important transcription factor for MMP-9 production (Bond et al., 1998, 2001; Moon et al., 2004). In various cell types, angiotensin II activates NF $\kappa$ B (Marui et al., 1993; Kranzhofer et al., 1999), and MMP-9 expression is mediated via this signalling pathway (Guo et al., 2008). NF $\kappa$ B activation could occur through phosphorylation and subsequent proteolytic degradation of inhibitory protein I $\kappa$ B (Ruiz-Ortega et al., 2006), but it was shown that there is weak degradation of I $\kappa$ B in response to angiotensin II, particularly in induction of MMP expression (Browatzki et al., 2005). Alternatively, angiotensin II could activate NF $\kappa$ B through phosphorylation of the p65 subunit at Ser 276 via AT1R (Kim et al., 2012).

Our previous study (Bundalo et al., 2015) demonstrated that FRD increased AT1R and ACE expression in ovariectomized rats and that oestradiol was able to revert these changes. Interestingly, in this study we demonstrated that the pattern of changes in MMP-9 expression due to FRD and oestradiol treatment is similar to those previously reported for AT1R and ACE expression (Bundalo et al., 2015). Considering previously mentioned facts (Marui et al., 1993; Kranzhofer et al., 1999; Bond et al., 2001; Moon et al., 2004; Guo et al., 2008; Okada et al., 2008, 2009, 2010), we found interesting to investigate changes in p65 phosphorylation at Ser 276 under the influence of FRD, and subsequent oestradiol treatment in the context of FRD. The results showed that FRD increased p65 phosphorylation, thereby activating NF $\kappa$ B, while E2 replacement therapy restored the initial level of phosphorylation in p65. A previous study implied that E2 inhibits NF $\kappa$ B activation by enhancing the inhibitor I $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) kinase level and/or by stabilization of I $\kappa$ B $\alpha$  (by decreasing its phosphorylation) (Wen et al., 2004; Xing et al., 2012). This is the first study demonstrating that E2 decreases phosphorylation of the p65 subunit of NF $\kappa$ B at Ser 276. NF $\kappa$ B plays a fundamental role in pathogenesis of insulin resistance and type 2 diabetes mellitus. Taking into account that NF $\kappa$ B, beside MMP-9, also controls many pro-inflammatory genes including cytokines (such as TNF- $\alpha$ , IL-6 and IL-8), adhesion molecules and chemokines (Cai et al., 2005; Shoelson et al., 2006; Wei et al., 2008), it is obvious that E2, by decreasing NF $\kappa$ B activation, displays its protective effect on the development of FRD-induced heart disorders. This effect of E2 on NF $\kappa$ B activation is in accordance with our previous study that demonstrates that E2 shows a protective effect on the development of cardiac insulin resistance in fructose-fed rats (Romcic et al., 2013).

The pattern of changes in p65 phosphorylation is similar to that obtained for MMP-9 under the same treatment. Our experiment demonstrated that early molecular changes in FRD-treated rats include elevated p65 phosphorylation at Ser 276, possibly as a result of increased AT1R expression. This phosphorylation activates NF $\kappa$ B, which consequently induces enhanced MMP-9 expression. Further studies are required to un-

equivocally confirm this signalling pathway. Should this diet regime be prolonged, the changes at the molecular level might promote adverse cardiovascular remodelling, which could result in development of various cardiovascular disorders. Obviously, this is the key point where changes at the molecular level have occurred but cardiac impairment has not yet emerged. An interesting finding was that the increase in MMP-9 expression and NF $\kappa$ B activation that occurred as a result of FRD was reversible with E2. This implicates that E2 shows a protective effect on the rat cardiac tissue by reducing the possibility of adverse ECM remodelling in the myocardium, which may be a result of the harmful effect of FRD.

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The authors declare no conflict of interest.

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