Uncoupling Proteins and Cardiac Apoptosis in High Fat Diet-Fed Male Rats

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ABSTRACT. This study aimed to assess impact of high fat diet (HFD) on cardiac apoptosis, and cardiac uncoupling proteins (UCPs) 2&3 expression. Twenty rats were fed either (45%) or (10%) fat diet for 6 months, then cardiac tissue expression of BAX, Bcl2, Fas, Fas-L (markers for apoptotic pathways) and UCP 2&3 were assessed. Results revealed enhancement of intrinsic and extrinsic cardiomyocyte apoptosis cascades in the myocardial tissue of HFD fed rats and down regulation of UCP2 and up regulation of UCP3 gene expression at 6 months. The present work highlights novel role for the uncoupling proteins in the experimental model of HFD feeding.

Keywords: Apoptosis; High fat diet rat model; Heart; uncoupling proteins (UCPs)

INTRODUCTION

Cardiovascular diseases are the primary cause of death worldwide (Smith et al. 2012); diet quality may be one of the most important factors associated with hypertriglyceridemia, hypertension, and many other cardiovascular risk factors (European Heart Network, 2011). In 1972, Kerr and colleagues introduced the concept of apoptosis and since then, the knowledge about that unique type of cell death has greatly expanded. Although apoptosis occurs in normal heart, it is important underlying mechanism in many cardiac diseases (Kung et al. 2011). Apoptosis cascade is initiated through either an intrinsic pathway which involves the mitochondria or extrinsic pathway that involves cell surface death receptors (Danial and Korsmeyer, 2004).

Uncoupling refers to a state in which nutrient fuels are oxidized but the energy is not linked to ATP synthesis but dissipated as heat. Uncoupling is mediated by (UCPs 1-4) located on the inner mitochondrial membrane, although presence of other proteins has been argued. Emerging functions of UCP2 and UCP3 are broadly different and findings are conflicting depending on the specific UCP and site of expression; up regulation of

UCP2 in pancreatic islets may develop insulin insufficiency in type 2 diabetes mellitus, while increased expression of UCP3 in skeletal muscle prevents development of insulin resistance (Chan & Harper, 2006). The implication of uncoupling proteins (UCPs) in cardiac apoptosis was not much investigated. So, further evidences concerning UCPs expression in various models and tissues are extremely needed. Up to our knowledge, no earlier works studied the alterations in cardiac UCPs in response to chronic intake of high fat diet (HFD), nor its relation to myocardial apoptosis. The current experimental study aimed to assess the alterations in cardiac expression of UCP 2&3 in experimental rat model of chronic HFD-induced cardiac apoptosis.

MATERIALS AND METHODS

Animals

Forty male Wistar rats weighing 150 -200 g were included in the current experimental study. The rats were obtained and maintained at the animal house, Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were housed under standard laboratory conditions of temperature, humidity and 12h light/dark cycle. Animals were treated in accordance with Guide for the Care and Use of Laboratory Animals (8th edition, National Academies Press), and the study was approved by the Research Ethical Committee, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia.

Experimental diets

Both standard and HFD were obtained from Research Diets Inc., New Brunswick, NJ, USA (Table 1).

Table 1. Standard and HFD formula.

Product details	Standard low fat diet D12450H		High fat diet, HFD D12451	
	gm %	Kcl %	gm %	Kcl %
Protein	19.2	20	24	20
Carbohydrate	67.3	70	41	35
Fat	4.3	10	24	45
Total		100		100
Kcl/gm	3.58		4.73	

Experimental design

Following one week of acclimatization, rats were randomly assigned to receive either the standard diet or HFD. These groups were as followings: Group I (n=10): control group, rats received the standard diet; Group II (n=10): rats received HFD.

Body weight (g) and oro-anal (OA) length (cm) were measured at the beginning to assess body mass index (BMI) (body weight (g) / the square of OA length (cm2) and at the end of the experiment. At 6 months, blood samples were collected following an overnight fast through the retro-orbital route. Sera were separated and maintained at −80Co for later measurements of total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), calcium and cortisol. Rats were sacrificed under diethyl ether anesthesia; hearts were excised, washed with normal saline and dried on filter paper. Heart specimens of the different groups were preserved in RNA1ater for the quantitative real-time RT-PCR assessments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Procedure of RNA extraction

Total RNA was isolated from the tissue using column purification technology using RNeasy Mini Kit (Qiagen). Separation of RNA was carried out according to the manufacturer's instructions (Qiagen). The tissue was lysed by the addition of 600 µl of lysis buffer (RLT) (provided in the kit) and β-mercaptoethanol was added and mixed by vortex then homogenized by IKA TIO basic. Equal volume of 70% ethanol was added to the lysate. The lysate was transferred to an RNeasy spin column attached to a 2 ml collection tube (provided in the kit). The column was centrifuged for 15 sec at 8,000 rpm (the flow through was discarded).

The cells were then washed with wash buffer 1 (RW1) (provided in the kit) and on column DNase digestion step was carried out. DNase was added and incubated for 15 min at RT. After incubation, the cells were centrifuged for 30 sec at 8,000 rpm. The cells were washed with wash buffer 1 (RW1) and centrifuged for 15 sec at 8,000 rpm. 500 µl of wash buffer 2 (RPE) (provided in the kit) was added and centrifuged for 2 min at 14,600 rpm. The RNA was then eluted by placing the column in a fresh collection tube and adding 30 µl of RNase free water (provided in the kit) followed by centrifugation at 14,600 rpm for 1 min. The RNA quantity and quality was measured with nanodrop.

Reverse transcription

Reverse transcription was prepared using cDNA reverse transcription kit (ImProm-II™ Reverse Transcription System cat no. A3800). The experimental RNA (up to 1µg) and 1 µl cDNA primer (Random Primer (0.5µg/reaction) were combined in Nuclease-Free Water for a final volume of 5µl per RT reaction. This step was performed on ice to avoid RNA degradation. Tubes was placed into a preheated 70°C heat block for 5 minutes then chilled on ice-water for at least 5 minutes. Each tube was spanned for 10 seconds in a microcentrifuge to collect the condensate and maintain the original volume. Reverse transcription reaction mix was prepared by combining the following components of the ImProm-II™ Reverse Transcription System in a sterile 1.5ml microcentrifuge tube on ice The 15µl aliquots of the reverse transcription reaction were mixed with each reaction tube (5µl). All the steps were performed on ice. The reaction was run on thermal cycler with the following cycling conditions: 25°C for 5 min, 42°C for 120 min and 70°C for 15 min.

Quantitative Real-time polymerase chain reaction (qRT-PCR)

The quantitative real-time polymerase chain reaction (qRT-PCR) was done using the KAPA SYBR® FAST qPCR Kit Master Mix (2x) Universal Cat no. KR0389. The experiment was done in duplicate for each sample in a 96 well plate which includes control samples, target genes, reference gene and non-template control for each gene PCR reactions were carried out using an ABI Step One Plus (Applied Biosystems) and started by initial denaturation at 95°C for 10 minutes, followed by 34 cycles at 95°C for 5 seconds, 65°C for 10 seconds, and 72°C for 15 seconds. Melting curve analysis was per-formed immediately after amplification by measuring the fluorescence of SYBR Green I during a temperature transition from 60°C to 95°C at 0.1 C/second.

Fluorescence data are converted into melting peaks using the Light Cycler Software Step One Software v 2.1 (Applied Biosystems). Quantitative values were obtained from the threshold cycle (Ct) number; the Ct is the threshold cycle at which the fluorescence curve reaches an arbitrary threshold. The comparative cycle threshold (CT) method used was also called 2 (-ΔΔCT) to determine the mRNA relative expression. First, the ΔCT value for each sample was calculated by determining the difference between the CT mean of the target gene and the CT mean of the endogenous control gene such as GAPDH. Then, ΔΔCT was determined by calculating the differences of the ΔCT value of the calibrator samples and the test samples. The fold change or the mRNA expression level was calculated according to this formula: 2 - ΔΔ CT (Table 2).

Table 2. Rats primers of apoptotic genes used in qRT-PCR.

Rat primers	Forward primer	Reverse primer
Bcl2	AGTGGGATACTGGAGATG	CTGGCTGTCTCTGAAGAC
BAX	CTGGACAACAACATGGAGC	CAGACGGCAACTTCAACTG
FAS	CTGATAGCATCTCTGAGG	CTGATAGCATCTCTGAGG
FAS-L	GACAACATAGAGCTGTGG	GACAACATAGAGCTGTGG
UCP2	TAACCATGGTGCGCACTG	TGGCAGGTAGCACCACA
UCP3	TCTACGACTCTGTCAAGC	GTGGTGAAGGTCCGATTT

Bcl2: B cell lymphoma 2 (anti-apoptotic), **BAX**: Bcl2-associated X protein (apoptotic activator), **FAS** receptor: member of death receptor family, **FAS-L**: FAS Receptor Ligand, **UCP2:** uncoupling protein 2, **UCP3:** Uncoupling Protein 3.

Statistical analysis

Statistical analysis of the data was carried out using SPSS for windows package version 20 (SPSS Inc., Chicago, IL, USA). The data were reported as the mean+/- standard deviation (SD) except for gene expression results, data were expressed as the mean +/- standard error of mean (SEM). The results were tested for normality using Shapiro-Wilk test. Data were analyzed by One-way Analysis of Variance (ANOVA) test to compare between groups. The Rq values of each gene were compared across all samples of four group using one-tailed student's "t"-test with unequal variance to calculate P value for identification of significantly expressed genes. The differences were considered statistically significant if $P < 0.05$.

RESULTS

BMI and biochemical analysis

At the end of the experiment, body weight, OA length and BMI were not significantly different between the study groups; In addition, serum levels of calcium, TC, TG, LDL-C, HDL–C and cortisol were not significantly differ among the four studied groups.

Quantitative Real-time polymerase chain reaction (qRT-PCR)

Apoptosis signaling pathways' markers: The components of both mitochondrial-dependent and extrinsic apoptotic signaling pathways were assessed in the current work through measuring expression of the anti-apoptotic Bcl2, pro apoptotic Bax, FAS and FAS-L genes in the cardiac specimens of all groups. At the end of 6 months, Bcl2 was downregulated while proapoptotic BAX expression was increased by HFD consumption. Vitamin D supplementation with the HFD increased Bcl2 expression, although did not reach significance and decreased Bax expression when compared to HFD group. The relative expression of FAS and FAS-L genes was significantly upregulated in HFD fed rats compared with control group and the level of both genes was decreased towards control levels when vitamin D was added to the HFD (Figures 1-4).
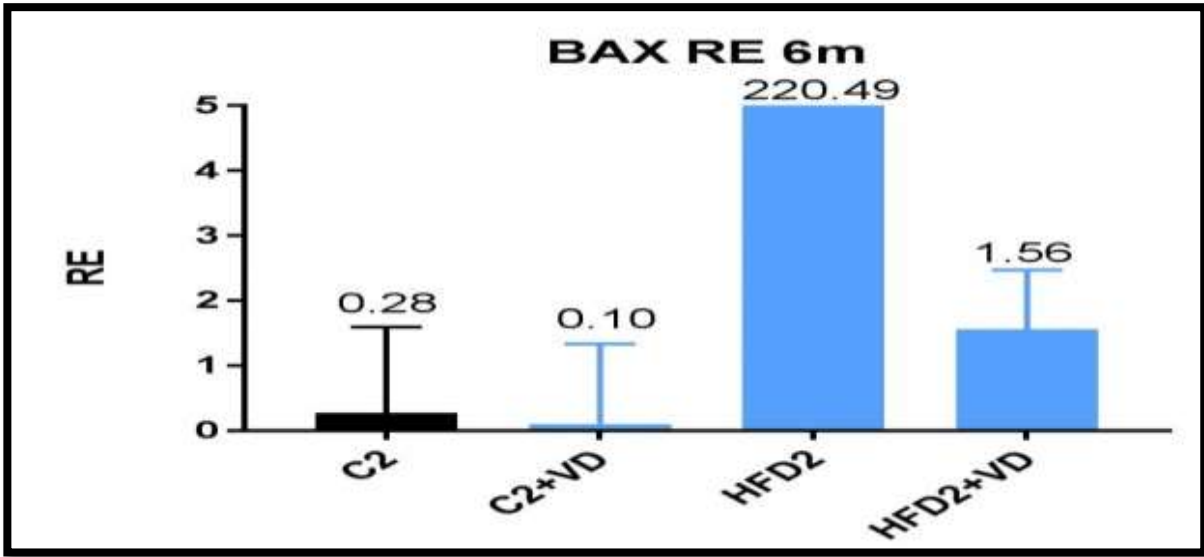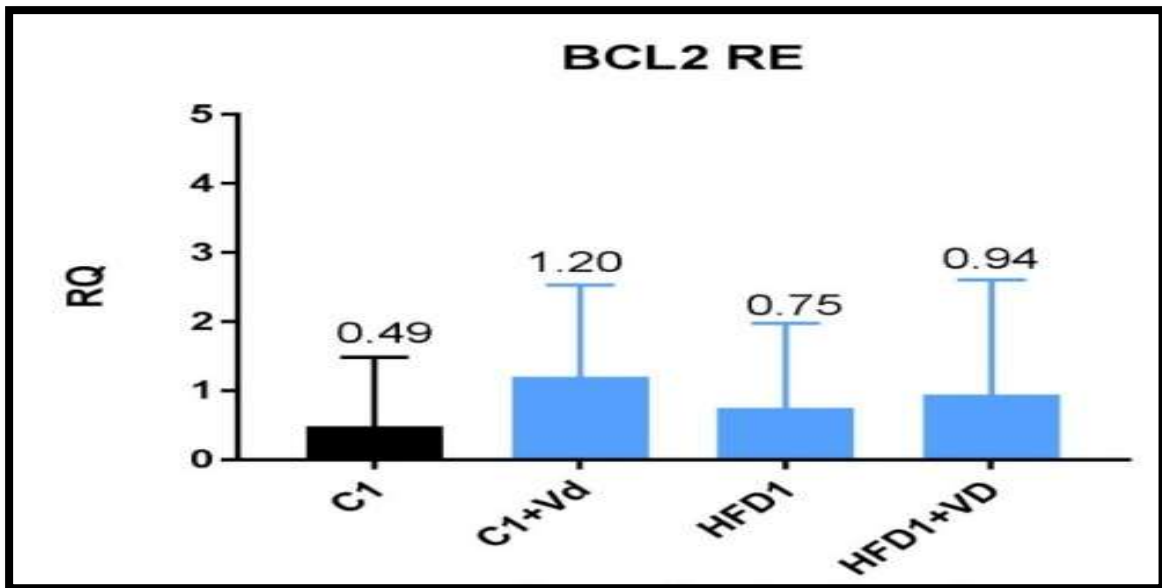
Figure 1. Relative expression pattern of BAX gene.
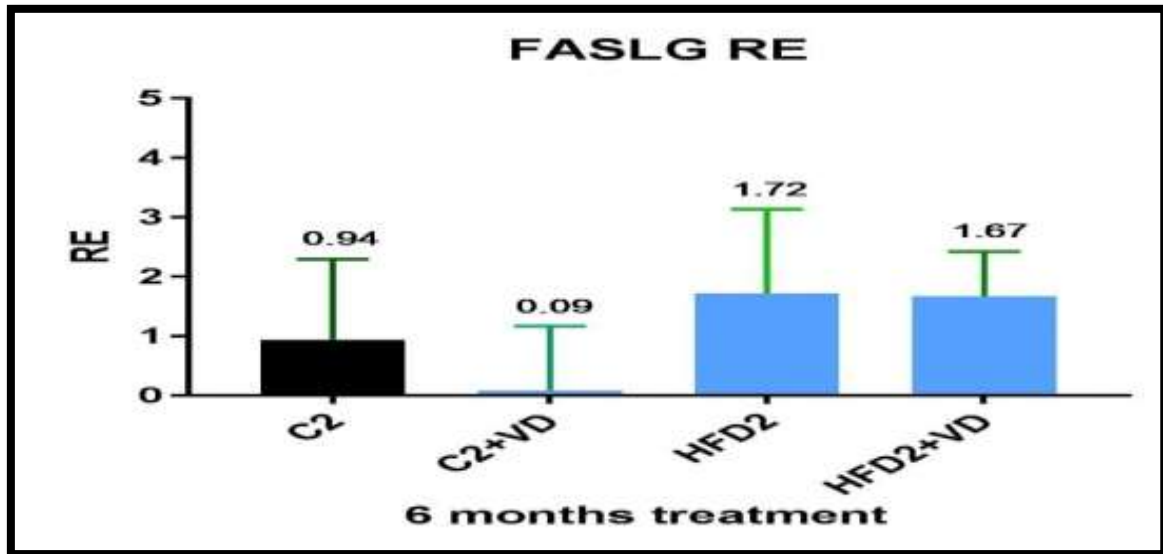

Figure 2. Relative expression pattern of BCL2 gene.

Figure 3. Relative expression pattern of FAS gene.
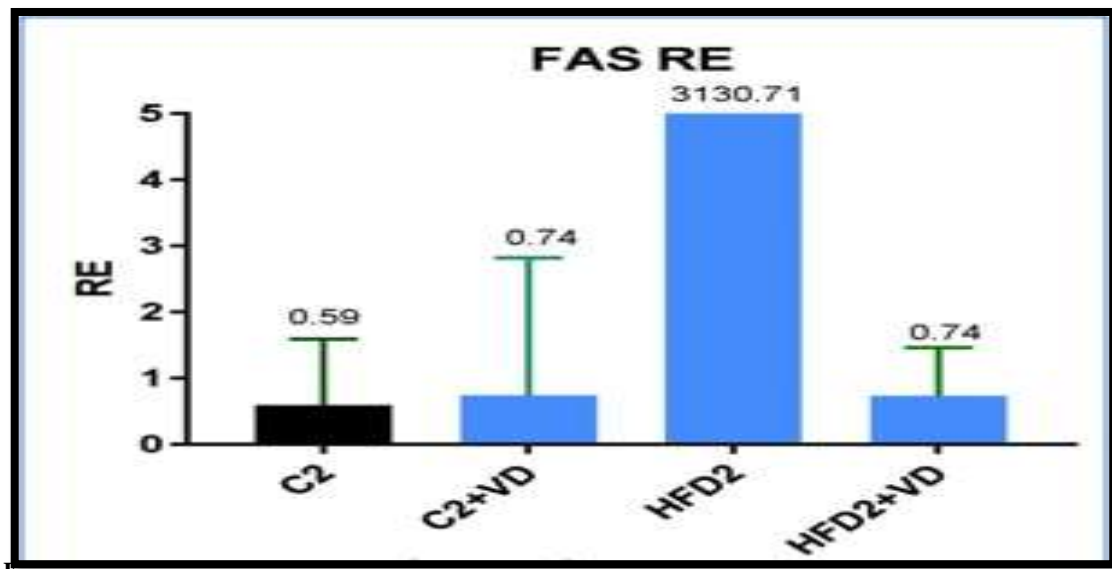

Figure 4. Relative expression pattern of FAS-L.

Uncoupling proteins

UCP2 expression was downregulated; while UCP3 expression was upregulated in HFD fed group with respect to control group at the end of the experiment. The expression of both UCP3 genes was decreased while of UCP2 gene was increased in HFD group supplemented with vitamin D3 compared to HFD fed group (Figures 5 and 6).
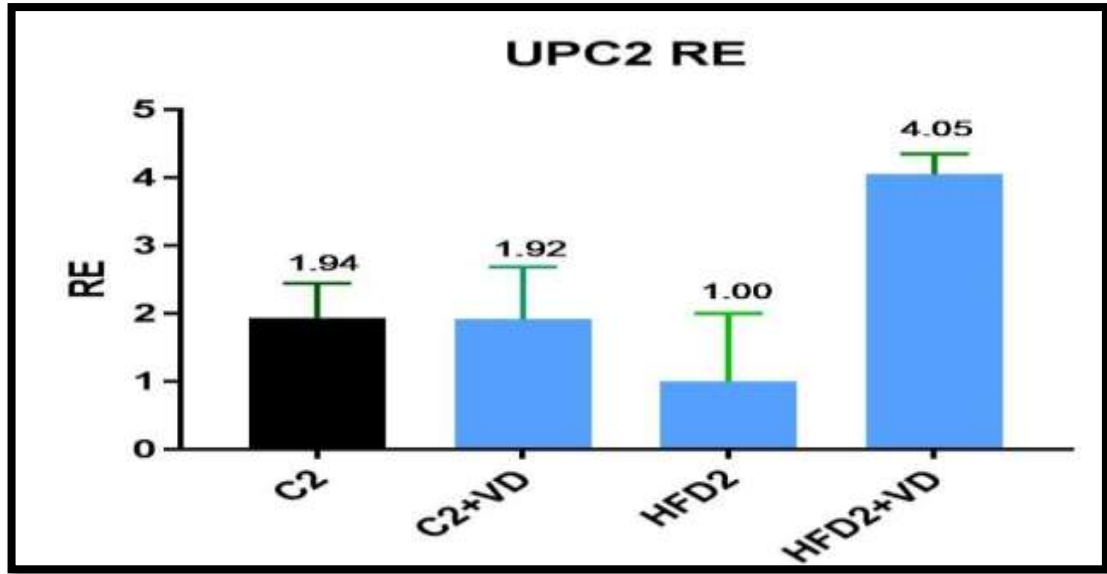
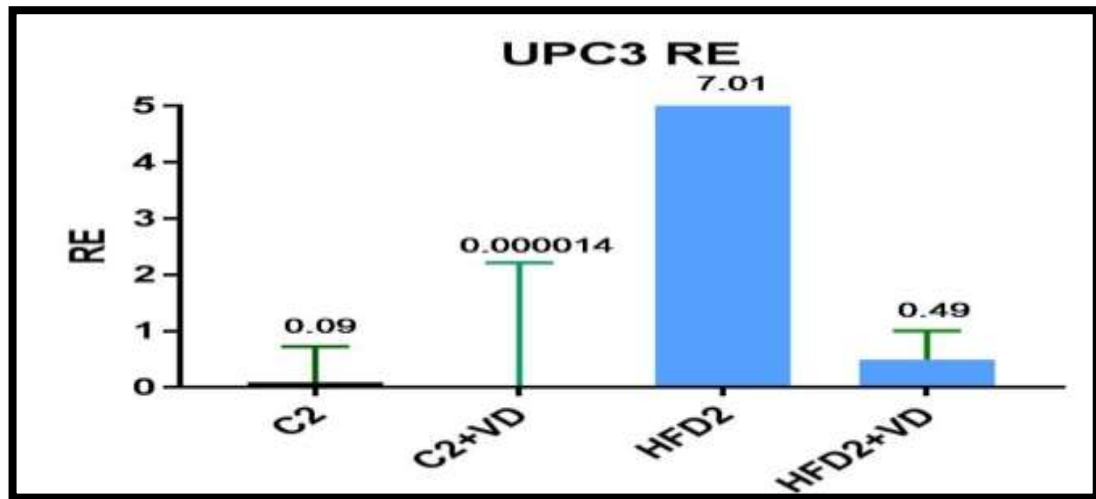**Figure 5.** Relative expression pattern of UCP2 gene.

Figure 6. Relative expression of UCP3 gene.

DISCUSSION

The current experiment comprised control group (standard diet 10% fat) and HFD group (45% fat). At the end of the experiment (6 months), no significant difference was observed between groups concerning BMI. These finding were in consistence with the previous work of Ramalho et al. (2017), where authors observed that rats fed a HFD (45% fat) for 15 weeks didn't significantly differ in their body weight from the control group.

Concerning lipid profile concentrations, the current observations clarified insignificant differences in lipid profile between the study groups following six months of HFD feeding. In accord with our finding, Opyd et al. (2018) showed that TC, LDL-C and TG levels were comparable between the control group and HFD fed group although the HFD group even received 1% cholic acid to stimulate lipid absorption. This could be attributed to the equal amounts of cholesterol in the experimental diet.

The results of current study revealed that there was an up-regulation of Bcl2 and down regulation of BAX in rats fed HFD. In consistency with the current findings, Cheng et al. (2013) showed induced-cardiomyocyte

apoptosis in hamsters fed with HFD (2% cholesterol) for 8 weeks with simultaneous increase in the levels of pro-apoptotic proteins BID and BAX, whereas the level of anti-apoptotic protein Bcl2 was decreased. Furthermore, Zhu et al. (2007) revealed elevation in the cardiac expression of BAX with decreased expression of Bcl2 in pigs fed with HFD for 12 weeks.

In the present study, HFD feeding increased Fas-L expression in the cardiac tissue as previously shown by Lin et al. (2017). The authors revealed that protein levels of cardiac Fas ligand, Fas receptors and Fas-associated death domain were significantly increased in rats fed 45% fat for 12 weeks.

The prominent finding of the present work is that significant downregulation of UCP2 but upregulation of UCP3 levels following 6 months of HFD feeding with respect to control group. The present study provides an evidence for the relation of cardiac apoptosis and UCPs expression in cardiomyocytes of HFD-fed rats. Marti et al. (2002) demonstrated that rats fed HFD for 65 days had significantly reduced UCP2 mRNA cardiac levels compared to control animals. However, Somoza et al. (2007) stated that mice which received HFD (45 % fat) for 8 weeks exhibited increase in UCP2 expression in their myocardial tissue compared to animals which received control diet (10% fat). Although the duration is not much different between Somoza's and Marti's studies, but the findings concerning UCP2 are controversial. Creus et al. (2016) observed that feeding rats with sucrose diet for 6 months to induce insulin resistance didn't significantly alter UCP2 in cardiomyocytes compared to control rats. In addition, in vitro studies performed by Li et al. (2010) revealed that primary cultured adult rat cardiomyocytes exposed to free fatty acids exhibited a dose-dependent increase in apoptosis and a significant increase in the level of UCP2 expression, while knocking down of UCP2 attenuated cardiomyocytes' apoptosis. The controversy concerning UCP2 gene expression between different previous studies and the current work could be attributed to the longer duration of the current experiment which lasted for 6 months.

In support to our findings, all previous studies that assessed cardiac UCP3 expression in response to HFD feeding (55-60% fat) revealed consistent results showing higher expression in comparison to control low fat feeding over different experimental periods ranging from 2-6 weeks (Cole et al. 2011, Liu et al. 2014, Cole et al. 2016). It's suggested that lipid peroxidation triggers the activation of UCPs (Murphy et al. 2003), consequently UCP 2&3 mediate some degree of proton leak within the mitochondria reducing the production of superoxide radicals (Douette & Sluse, 2006). Overexpression of UCPs in response to HFD might seem a beneficial defense process to decrease reactive species production and guard against cardiomyocyte apoptosis (Bodyak et al. 2007). It was observed in many clinical studies and experimental models of heart failure that UCP 2&3 are often decreased. It isn't surprising, because reduced UCPs expression would enhance coupling between citric acid cycle and ATP synthesis, increasing energetic efficiency which is important for cardiomyocytes although this might give the chance for greater production of reactive oxygen species (Hesselink & Schrauwen 2005, Neubauer 2007). It seems that the factors contributing to regulation of UCPs genes are complicated; over-expression will guard against oxidative stress while under expression would enhance energy resources for the myocardial cells. Indeed, more studies are needed to investigate for the factors influencing the myocardial UCPs gene expression under different circumstances.

CONCLUSION

The current work highlighted the effect of long term HFD consumption on cardiac expression of UCP 2&3 and enhancement of both intrinsic and extrinsic cascades of cardiomyocyte apoptosis. The current observations expanded our knowledge concerning the mechanisms underlying myocardial apoptosis in chronic model of HFD-fed rats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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