ISSN NO. 2320-5407

Journal homepage: http://www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH

RESEARCH ARTICLE

Impact of chronic stress and exercise on inflammatory and oxidative stress biomarkers, and apoptotic genes in rats fed high fat diet.

Nisreen Mansour Abo-elmaaty Omar[1], Amany Atwa[2], Dalia Saleh[3]

1. Department of Medical Physiology, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
2. Department of Medical Biochemistry, Faculty of Medicine, Mansoura University, Mansoura, Egypt.
3. Department of Anatomy, Faculty of Medicine, Mansoura University, Mansoura, Egypt.

Manuscript Info

Manuscript History:

Received: 15 October 2015
Final Accepted: 22 November 2015
Published Online: December 2015

Key words:

Obesity, High fat Diet, Chronic stress, MCP-1 gene, p53 gene, Bcl-2 gene.

Corresponding Author

Nisreen Mansour Abo-elmaaty Omar
(nisreenomar@hotmail.com)

Abstract

Aim: This study aimed to investigate the impact of combination of exposure to chronic stress and high fat diet on the inflammatory and oxidant stress milieu together with the effect on expression of p53, Bcl-2 in adipose tissue and vascular tissue.

Methods: Forty male Sprague-Dawely rats were divided equally into 5 groups: (**ND**) fed normal chow diet, (**ND-S)** fed normal chow & exposed to different stressors for 12 weeks, (**HFD)** fed high fat diet, (**HFD-S)** fed high fat diet & exposed to stress and (**HFD-S-E)** fed high fat diet with exposure to stress and had swimming exercise sessions.

Results: Serum lipid profile was significantly disturbed in HFD-S rats but only Fasting blood glucose and total serum cholesterol were elevated in stressed chow fed rats. Serum insulin, cholesterol, TGs level decreased in HFD-S-E group compared to HFD-S rats. Oxidative markers were elevated in HFD & HFD-S groups compared to ND group. Adipose tissue mRNA level of MCP-1 & p53 genes was increased in both ND-S and in HFD-S while its mRNA levels of BcL-2 gene was significantly reduced in HFD-S rats than in HFD group. BcL-2 mRNA was increased in HFD-S-E rats vs. HFD-S group. Sections from visceral adipose tissue and heart from HDF-S rats showed some degenerative changes and inflammatory cell infiltration. p53 Immunostaining was positive in adipose and heart tissue from stressed groups.

Conclusion: High fat diet feeding is associated with low grade inflammatory state, insulin resistance, enhanced oxidative stress and disturbed balance between proapoptotoic and antiapoptotic genes. Chronic stress per se seems to have its deleterious effect. However, combination of HFD & stress may potentiate the hazardous effect of each other. Moreover exercise alone may not be sufficient to reverse HFD-S induced hazardous effects.

INTRODUCTION

Obesity is a world-wide problem with its recognised burdens on different body systems especially the cardiovascular system. On the other hand, our daily exposure to stress is an accepted fact in our life. The relation between stress and obesity has attracted attention from researchers in a trial to clarify which is the cause and which is the consequence (Dallman et al., 2003). Animal studies have produced conflicting results as to whether exposure to stress leads to adiposity or not. Thus, restraint stress with normal diet caused weight loss in wild obesity-prone mice while the mice adapted to cold water avoidance test showed no change in weight (Michel et al., 2005). While sustained stress by exposure to cold for 1 h increases weight but only with high fat diet (Kuo et al., 2008).

On the other hand, the increased risk of cardiovascular diseases (CVD) with obesity is known to be correlated with traditional risk factors as hyperlipidaemia, diabetes and hypertension (Eckel etal., 2002). However, the mechanisms that could clarify the link between obesity and CVD are still not fully understood. Studies have tried to elucidate such mechanisms through identification of biomarkers resulting from obesity and independently enhance susceptibility for CVD. Inflammation and oxidative stress are thought to be involved in this association between obesity and CVD. Abnormal fat accumulation has been linked to inflammatory changes such as recruitment of macrophages and activation of endothelial cells, both lead to vascular diseases (Curat et al., 2004). In addition, fat accumulation has been found to be associated with an increased oxidative stress that by its turn enhances proinflammatory state that dysregulate adipocytokines released by adipocytes and hence leads to metabolic disorders as insulin resistance (Furukawa et al., 2004). Thus, a recent approach in this field has indicated that biomarkers for inflammation and oxidative stress can prove useful in the prediction of CVD in the obese as non-traditional biomarkers. Another inflammatory biomarker linking obesity and CVS is ***monocyte chemoattractant protein 1*** (***MCP-1***). In vitro studies have shown that MCP-1 expression was induced in murine adipocytes by metabolic and inflammatory mediators such as insulin and TNF-α and that deletion of MCP-1 in mice susceptible to atherosclerosis has led to a substantial reduction in the formation of atherosclerotic lesions (Martinovic et al., 2005). Also, assessment of oxidative stress can be done indirectly by measuring oxidised products of lipids, proteins and DNA. Of these, ***oxidised low density lipoprotein*** **(oxLDL)** has been shown to be cytotoxic and is readily taken up by macrophages and thus strongly contributes to the development of atherosclerotic lesions (Guo et al., 2001). Studies in guinea pigs have demonstrated that OxLDL induced cellular damage and irregular electrical activity in ventricular myocytes (Zorn-Pauly et al., 2005), indicating the potential role of oxLDL in promoting CVD. Another sensitive indirect biomarker for oxidative stress is ***F2-isoprostanes,*** products of lipid peroxidation (Gross et al., 2005). Also, glutathione peroxidase and its substrate glutathione are a major antioxidant defence system against increased oxidative stress, demonstrating strong antioxidant and antiatherosclerotic properties protecting the vascular endothelium from oxidative stress.

Hypertrophied adipocyte-derived cytokines such as tumor necrosis factor TNF-α have been reported to mediate insulin resistance in obesity. Considering that TNF_α is relevant to both cell growth and metabolic events and its cytotoxic effects were demonstrated to be mediated partly by ***p53*** activation, it was speculated that ***p53*** could be involved in metabolic deterioration associated with obesity and insulin resistance (Rokhlin et al., 2000). On the other hand, the family of caspase enzymes that is distributed in the cytoplasm, mitochondrial inter-membrane space, and nuclear matrix involved in apoptosis which represents a fundamental mechanism for the homeostasis of mammalian tissues (Chandra et al., 2001). Another large protein family ***B-cell lymphoma 2 (Bcl-2)*** proteins, is also involved in this process which regulate mitochondrial permeability processes and constitute a key point for the mitochondrial pathway of apoptosis. Bcl-2 is well-known to be a potent prosurvival advocate with antiapoptotic effects (Adams & Cory, 1998).

To this point, the objective of this work is to study whether exposure to chronic stress while eating high fat diet would further aggravate the inflammatory and oxidant stress milieu as measured by inflammatory and oxidative stress biomarkers. Moreover, the effect of such combination on expression of p53, Bcl-2 in adipose tissue and vascular tissue will be investigated. A third aim was to investigate the effect of such combination on histological structure of adipose tissue.

Methods & Materials:

Animals & Experimental groups

Male Sprague-Dawely rats were obtained from and housed at animal house unit in Medical Experimental Research Centre (MERC), Mansoura Faculty of Medicine. After the age of weaning (~ 25 days) rats were fed normal rat chow until they reached sexual maturity (by the age of 35-40 days). Then, rats were randomly allocated into two dietary regimens: normal chow fed rats (no. =16) that were further divided into 2 groups (each = 8 rats): (**Group I)** non stressed normal diet (ND); (**Group II)** stressed normal diet group (ND-S). High fat diet fed rats (no. =24) that were further divided into 3 groups (each = 8 rats): (**Group III)** non-stressed high fat diet group (HFD); (**Group IV)** stressed high fat diet group which received high fat feeding in association with application of stress protocol (HFD-S); (**Group V)** stressed HFD group that were fed high fat diet in association with stress protocol and put to exercise in the last 8 weeks (HFD-S-E). High fat diet group had high fat feeding for about 12 weeks (High fat diet comprised of 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal respectively (Srinivasan et al., 2005).

Stress Protocol

Stressed ND and HFD groups were subjected to a protocol of chronic stressors that were applied daily for 12 weeks. The stressors were either 1) cage tilt at 40 degrees for the whole night, 2) water deprivation over night followed by empty bottles for 1 h, 3) sleep deprivation by keeping light on overnight, 4) wetted bedding (Michel et al. 2005). The stressors were applied in a rotatory way. The rats were weighted regularly to check the weight gain. After 12 weeks, the animals were sacrificed for tissue and blood sample collection.

Exercise protocol

The rats in the exercise group were subjected to period of daily continuous swimming in individual tanks filled with water maintained between 28°C and 32°C. The animals swam for 15 min daily for 2 days to adapt to the protocol. Then, the swimming period was increased to 30 min with a weight of 5% body weight attached to the tail of each rat (Estadella et al. 2004). The exercise protocol continued for 5 d/wk for 8 weeks.

- **Collection of *Blood samples & Tissue specimen***

At the end of the experimental period, the animals were fasted overnight for 8 hours and were sacrificed under pentobarbital (50 mg/kg body weight) anesthesia. Blood samples were drawn from the abdominal aorta, centrifuged then sera were separated out and frozen at −20 °C for biochemical measurement. Epidydmal and visceral adipose tissue were collected and snap frozen in liquid nitrogen for gene expression. Also, samples from thoracic aorta and heart tissue were collected in the same way.

A) Biochemical measurements

***i*- Assay of serum corticosterone:**

Serum corticosterone level was determined using a commercially available enzyme immunoassay kit (IDS, Boldon, UK). Serum samples were assayed in duplicate. All procedures were performed according to the manufacturer's instructions. Final values were determined by averaging the results of duplicated samples.

***ii*- Assay of serum glucose, insulin & lipid profile:**

Serum glucose was determined colorimetrically based on Glucose Oxidase Method (Trinder 1969) using Span Diagnostic kits (Surat, India) and results were reported as mg/dL. Serum insulin level was estimated using ELISA Kit (Hwang et al., 1985). Serum total cholesterol, triglycerides and HDL-cholesterol levels were analyzed enzymatically using kits obtained from Randox Laboratories Limited, Crumlin, United Kingdom (UK) according to method of (Allain et al., 1974, Werner et al., 1981 & Burstein et al. 1970; respectively).

Calculation of Homeostasis Model Assessment of Insulin Resistance index (HOMA-IR):

HOMA-IR index was calculated according to the equation (Pickavance et al., 2005)

$$\frac{\text{Fasting insulin level } (\mu U/mL) \times \text{fasting plasma glucose (mmol/L)}}{22.5}$$

***iii*- Determination of Oxidative stress biomarkers (MDA, ox-LDL, iso-F2):**

- ***Malondialdehyde (MDA):***
  Serum malondialdehyde was measured by the method of Ohakawa *et al*. (1997). Lipid peroxidation generates peroxide intermediates which upon cleavage release MDA which reacts with Thiobarbituric Acid (TBA). The product of the reaction is a colored complex, which absorbs light at 532 nm
- ***Oxidized LDL:***
  Serum samples were analyzed using Glory OxLDL kits (USA) that work upon a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was utilized to evaluate the levels of OxLDL in samples. The assays were performed according to the manufacturer's instructions in duplicate.
- ***8-isoprostane (8-iso PG-F2α):***
  Serum 8-isoprostane levels were detected to reflect levels of systemic oxidative stress using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) using mouse monoclonal antibody to 8-isoprostane and the plate was read at 402 nm (Badr & Abi-Antoun, 2005).

B) Study of gene expression in visceral adipose tissues:

- Expression of monocyte chemoattractant protein 1 (MCP-1) mRNA, p53 mRNA and Bcl-2 mRNA:

Amplification of cDNA by PCR:

Epidydmal adipose tissues samples were collected, and total RNA was extracted using RNeasy mini kit (Qiagen, cat no. 74104) as described in the manufacturer's instructions. The quality of extracted RNA was tested by measuring the absorbance at 260 nm using the Nanodrop spectrophotometer, NanoPhotometer® P-Class (Implen, Germany). The absence of RNA degradation was confirmed by electrophoresis on a 1.5% agarose gel electrophoresis containing ethidium bromide. First-strand cDNA was generated by using Maxima First Strand cDNA Synthesis Kit

(Thermo Scientific, U.S.A). Gene specific primers were purchased from BIOSEARCH TECHNOLOGIES (South McDowell Blvd, Petaluma, Canada). The sequences of the oligonucleotide primer sets for the PCR amplification of rat mRNAs were designed according to published sequences **(Table 1).**
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping control gene to verify the quality of cDNA. Amplification was done using Emerald Amp GT PCR Master Mix provided by Takara, Japan, code No. RR310A. Thermal cycling reaction was performed using a PTC-200 thermal cycler (MJ Research, Essex, UK). Thermocycling was performed according to the following profile:
initial denaturation at 94°C for 3min, followed by 35 cycles of denaturation at 94°C for 1 min.; annealing at (55°C for 45 sec), (60°C for 2min.), (58°C for 30 sec) and (55°C for 30sec) for MCP-1, P53, Bcl-2 and GAPDH respectively; and extension at 72°C for 1 min. Followed by a final extension 72°C for 10 minutes. The PCR products were electrophoresed on 2% agarose gel and DNA was visualized with ethidium bromide staining under UV illumination.

- **Table (1): Sequence of all primers used:**

Gene	Primer sequence	PCR product size (bp)	Reference
MCP-1	F: 5'- ATC CCA ATG AGT AGG CTG GAG AGC -3' R: 5′- GGT GGT TGT GGA AAA GGT AGT GG -3'	**279**	**(Ransohoff et al., 2002)**
p53	F: 5' -TCT GTC ATC TTC CGT CCC TTC TC-3' R: 5′-AAC ACG AAC CTC AAA GCT GTC CCG-3'	**547**	**(Soussi et al., 1988)**
Bcl-2	F: 5′-CCT GCC CCA AAC AAA TAT GAA AAG -3′ R: 5′-TTG ACC ATT TGC CTG AAT GTG TG-3'	**174**	**(Keshavarz et al., 2013)**
GAPDH	F: 5'-GTC TTC ACC ACC ATG GAG-3′ R: 5′-CGA TGC CAA AGT TGT CAT G-3′	**211**	**(Ornellas et al., 2002)**

- **MCP-1**= Monocyte chemoattractant protein 1
- **p53=** Tumor suppressor gene
- **Bcl-2=** B-cell lymphoma -2
- **GAPDH**= Glyceraldehyde 3-phosphate dehydrogenase

C) Histological and immunohitstological examination
Tissue specimens from the adipose tissue and the hearts were harvested then fixed in 10% buffered neutral formalin for at least 3 days prior to further processing for paraffin sectioning. The tissues were washed twice with saline, dehydrated in a series of ascending concentrations of alcohol and cleared with xylene. The specimens then were embedded in paraffin and cut at 4-µm thick. The sections from each paraffin block were stained with Hematoxylin and Eosin (H&E) for histopathological assessment and p53 immunoperoxidase stain. The sections then were examined and photographed using Olympus light microscopy (Olympus BX-50, Tokyo, Japan) and photographed with a high-resolution digital camera (Olympus LC20- Japan).

p53 immunohistochemistry
Sections of 4-µm thick of formalin-fixed paraffin-embedded tissue were deparaffinized, rehydrated through graded alcohols, then transferred into 0.01 m citrate buffer (pH 6.0), boiled on a hot plate for 20 minutes, cooled for 40 minutes, and washed in distilled water for 5 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 minutes. Sections were then incubated with monoclonal p53 antibody (Clone BP53 12-1, Biogenex, CA) at a dilution of 1:40 for 30 minutes at room temperature. Sections were then incubated with a biotinylated rabbit anti-mouse IgM (Dako E465) diluted 1:80 in PBS for 20 minutes at RT, followed by streptavidin conjugated with horseradish peroxidase substrate, composed of 3,3' diaminobenzidine 0.01 gm, Triton solution (0.5%) 15 mL, and H_2O_2 (30%) 0.01 mL, was then applied to sections for 10 minutes. Sections were then stained with hematoxylin and mounted in Permount (Batheja et al., 2000).

Statistical Analysis

The results were expressed as mean ± SEM. Statistical analysis of data among the groups was performed by using analysis of variance (ANOVA) to determine the level of significance. P values less than 0.05 ($p<0.05$) were considered significant.

Results:

1. EFFECT ON BODY WEIGHT & SERUM CORTICOSTERONE LEVEL:

The high-fat diet induced the expected overweight model. As shown in Table (2), final body weights of the rats fed on high-fat diet were statistically greater in comparison with the standard chow-fed rats. Exposure of rats fed standard chow diet to chronic stress induced a significant gain in body weight, though less when compared to the effect of high fat diet ($p<0.05$). There was no significant difference between BW of HFD, stressed HFD or HFD-S-E.

Regarding serum corticosterone level, as an index of stress, exposure to stress induced a significant increase in serum corticosterone levels in both ND & HFD groups ($p<0.0001$). Feeding rats high fat diet increased serum corticosterone further ($p<0.01$ HFD-S vs. ND-S rats) whereas exercise eliminated such increase as shown in Table 2.

TABLE (2): Effect of high fat diet (HFD) alone, HFD with stress (HFD-S), HFD with stress and exercise (HFD-S-Ex) on body weight and serum corticosterone level as compared to normal diet group (ND)

	NORAMAL DIET GROUP		HIGH FAT DIET GROUP		
	ND	STRESSED (ND-S)	HFD	HFD-S	HFD-S - E
BODY WEIGHT (gm)	266.3 ±4.2	288.8* ±11.9	424.4*** ±11.6	438.8 ±10.9	442.5 ±11.3
SERUM CORTICOSTERONE (ng/ml)	1.56 ± 0.11	2.89**** ± 0.12	3.25**** ± 0.24	4.04$^{\#\#}$ ±0.30.	3.55 ± 0.39

Each group = 8 rats

All values are mean ± SEM (one way ANOVA).

p* < 0.05, p**<0.01, p*** <0.001, p**** <0.0001; vs. ND group.

p$^{\#\#}$ <0.01 , HFD-S vs. ND-S.

2. EFFECT ON BIOCHEMICAL PARAMETERS:

As table 3 shows, exposure to chronic stressors has significantly increased blood level of glucose and cholesterol only when compared with non stressed ND group (p=0.01, 0.002 respectively), while induced a significant decrease in HDL (p=0.0001). Feeding rats with high fat diet induced a significant increase in blood glucose and insulin levels, compared to ND or ND-S groups. Stress and HFD induced a greater increase in glucose and insulin blood levels compared to HFD alone ($p<0.01$, 0.001 respectively). Exercise reduced the increase in blood glucose level but it did not reach significance while it reduced significantly blood insulin level as compared to HFD-S group (Table 3).

As shown in table 3, HFD induced a significant increase in all parameters of lipid profile, namely TC, TG, LDL while it induced a great reduction in HDL. The increase in TC, TG and reduction in HDL was further aggravated by exposure to stress with HFD but that effect was absent with LDL (table 3). Exercise reduced significantly the increase in TG & TC only when compared with HFD-S group (p= 0.002, 0.009 respectively) while the decrease in LDL level did not reach significance when compared with HFD-S group. Also, exercise has increased the level of HDL but such increase was not insignificant.

HOMA-IR value was significantly increased in HFD group when compared with ND group and ND-S group (Fig.1). In HFD-S group, HOMA-IR was significantly greater than in HFD group, but it was reduced in HFD-S-E when compared to stressed HFD group (Fig.1).

TABLE (3): Blood level of glucose, insulin and lipid profile parameters in normal diet group (ND) stressed normal diet (ND-S), non- stressed high fat diet group (HFD), high fat diet with stress (HFD-S) and HFD exposed to stress and exercise (HFD-S-E).

	NORMAL DIET GROUP		HIGH FAT DIET GROUP		
	ND	**ND-S**	**HFD**	**HFD-S**	**HFD-S- E**
GLUCOSE (mg/dl)	84.49 ± 2.57	99.79* ± 4.85	158.4*** ±10.5	204.3$^{\#}$ ±16.5	179.6 ± 9.26
INSULIN (µu/ml)	2.51 ± 0.33	2.73 ± 0.28	11.39**** ± 0.97	19.68$^{\#\#\#}$ ± 0.52	12.29$^{\$\$\$}$ ±0.99
TOTAL CHOLESTEROL (mg/dl)	83.91 ± 3.43	145.1** ±15.97	206.8**** ±10.01	255.6$^{\#\#}$ ±9.33	187.6$^{\$\$}$ ±20.
TRIGLYCERIDES (mg/dl)	54.14 ± 3.43	59.68 ± 3.9	169.0**** ± 3.59	204.4$^{\#\#}$ ± 9.19	168.8$^{\$\$}$ ± 3.63
LDL (mg/dl)	34.75 ± 2.23	38.03 ± 2.27	162.11**** ± 5.04	155.9 ± 4.34	146.88 ± 4.07
HDL (mg/dl)	46.60 ± 2.11	34.94*** ± 0.61	20.54**** ± 1.38	15.72$^{\#}$ ± 1.10	18.03 ± 2.02

Each group = 8 rats
All results are expressed as mean ± SEM (one way ANOVA).
P* < 0.05, P**<0.01, P*** <0.001, P**** <0.0001; VS. ND group.
P$^{\#}$ < 0.05, P$^{\#\#}$ <0.01, P$^{\#\#\#}$ <0.001; VS. HFD group.
P$^{\$\$}$ <0.01, P$^{\$\$\$}$<0.001 ; VS. HFD-S group.

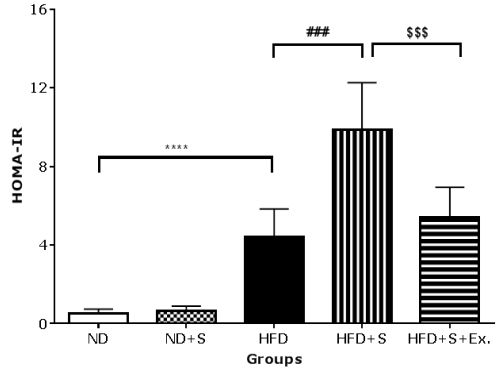


Fig. 1. HOMA-Insulin Resistance in normal diet group (ND), stressed normal diet group (ND-S), high fat diet group (HFD), stressed high fat diet group (HFD-S) and in stressed high fat diet with exercise group (HFD-S-E). Each group =8 rats. All results are expressed as mean ± SEM. P$^{\#\#\#}$, $^{\$\$\$}$ <0.001, p****<0.0001 (one way ANOVA).

***3. EFFECT ON OXIDATIVE STRESS PARAMETERS; MDA, OXLDL & F_2 ISOPROSTANE*:**

Fig.2. shows that either chronic stress or HF feeding has induced a significant increase in the level of MDA as compared to ND rats ($p < 0.001$, 0.0001 respectively). Stressed HFD group had greater significant increase in MDA level when compared to non-stressed HFD group. Although the level of MDA was significantly reduced in stressed HFD group with exercise as compared to HFD-S group ($p<0.01$) but it was still greater than that in HFD alone ($p< 0.001$).

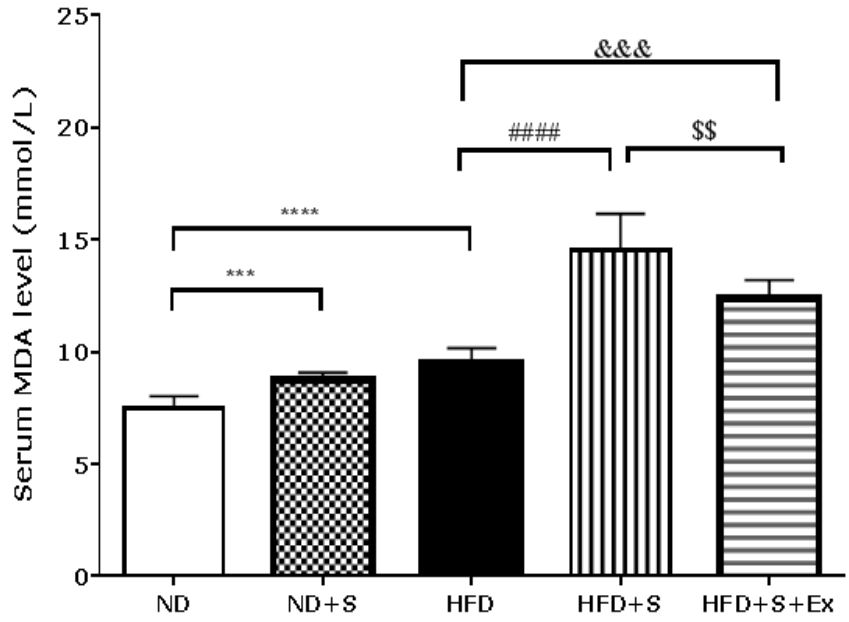


Fig.2. Serum Malonaldehyde (MDA) level (mmol/L) in normal diet group (ND), stressed normal diet rats (ND-S), high fat diet group (HFD), stressed high fat diet group (HFD-S), and in stressed high fat diet with exercise (HFD-S-E). Each group =8 rats. All results are expressed as mean ±SEM. $p^{\$\$}<0.001$, p^{***}; $p^{\&\&\&}<0.001$, p^{****}; $^{\#\#\#\#}<0.0001$ (one way ANOVA).

Regarding oxLDL, there was no significant difference between ND & ND-S groups whereas its level was increased markedly in HFD group when compared to either ND or ND-S group ($p<0.0001$). Its level was greater in stressed-HFD as compared with HFD group ($p<0.01$). Exercise lowered its level significantly in HFD-S-E group when compared to stressed HFD group to be comparable with with non-stressed HFD (see Fig. 3).

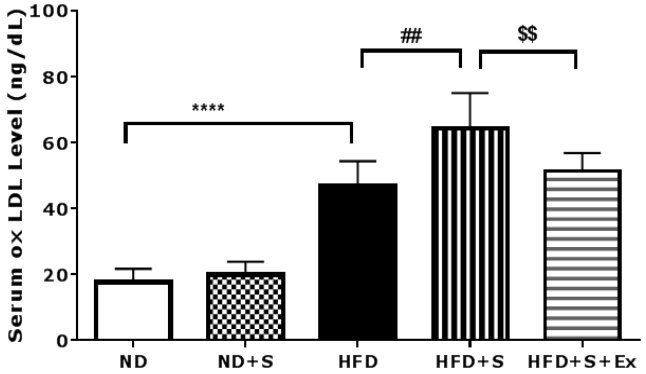


Fig.3. Serum level of oxDL (ng/dl) in normal diet group (ND), stressed normal diet group (ND-S), non stressed high fat diet group (HFD), stressed high fat diet group (HFD-S) & in stressed HFD with exercise (HFD-S-E). Each group =8 rats. All results are expressed as mean SEM. $p^{\#\#;\$\$}<0.01$; $p^{****}<0.0001$ (one way ANOVA).

As shown in Fig.4., F_2 Isoprostane levels demonstrated insignificant difference between normal diet groups while there was high significant increase in HFD as compared to ND & ND-S groups ($p<0.0001$, 0.01 respectively). HFD with stress further increased the level of F_2 Isoprostane as compared to HFD only. A significant decrease in F_2 Isoprostane level was observed in HFD-S-E compared to stressed HFD ($p=0.0006$) while there was insignificant difference with non-stressed HFD group (Fig.4).

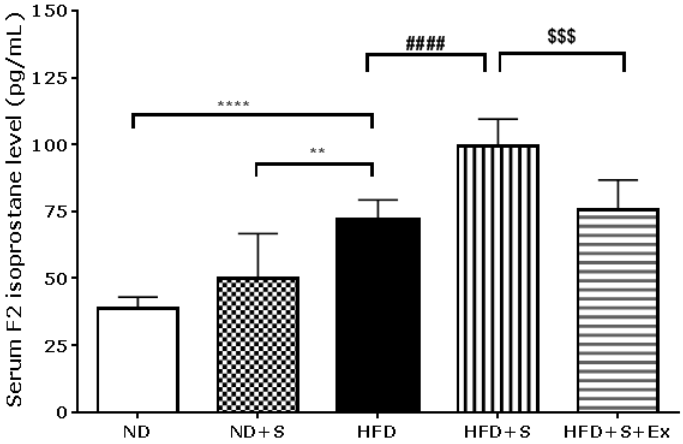


Fig. 4. Serum level of F_2 Isoprostane (pg/ml) in normal diet group (ND), stressed normal diet group(ND-S), high fat diet group (HFD), stessed high fat diet group (HFD-S), and in stressed high fat diet with exercise group. Each group = 8 rats. All results are expressed as mean ±SEM. $p^{**}<0.01$,$p^{\$\$\$}<0.001$, p^{****}, $^{####}<0.0001$ (one way ANOVA).

4. EFFECT ON MCP-1(AS AN INFLAMMATORY BIOMARKER)

Images in (Fig.5.a) show expression analysis of MCP-1mRNA from visceral adipose tissue, heart and thoracic aorta extracted from different groups. As seen in Fig. (5.b), there was significant increase in its expression in stressed ND group as compared to non-stressed ND group. Also, there was greater increase in its expression in non-stressed HFD than in normal diet groups (non-stressed, $p<0.0005$ and stressed, $p<0.05$). A significant increase in stressed HFD was observed compared to non-stressed HFD ($p<0.005$). Its expression was reduced significantly in Stressed HFD with exercise compared to the stressed-HFD group (Fig.5.b).

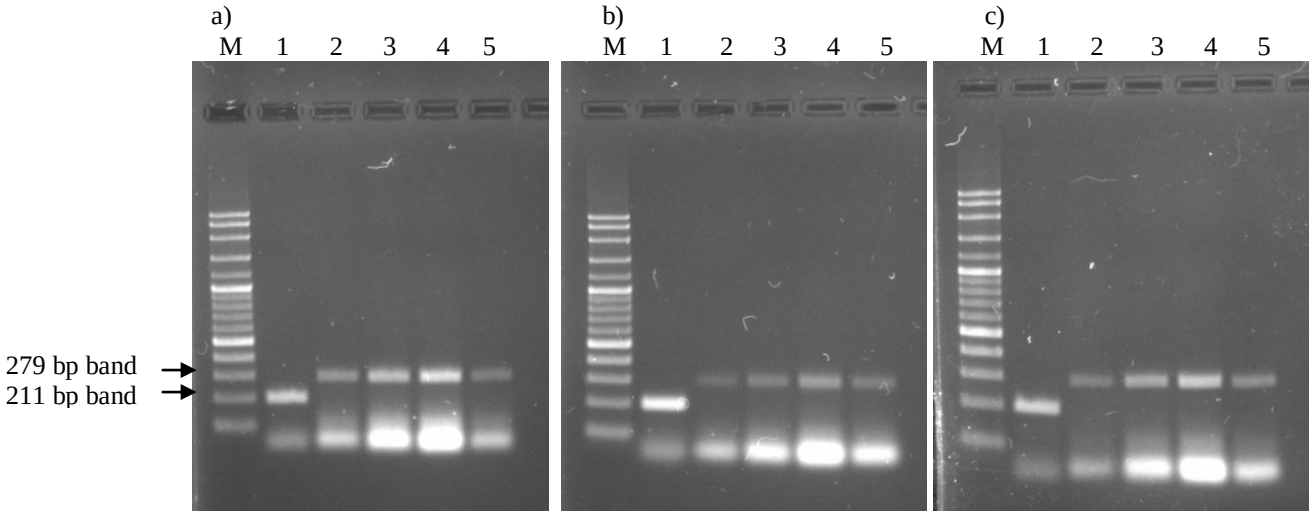


Fig 5.a. Expression analysis of MCP-1 mRNA from a) visceral adipose tissues, b) heart & c) aorta by RT-PCR. Lanes 2: samples from rats on normal diet, Lanes 3: samples from stressed rats on normal diet; Lanes 4: samples from stressed rats on high fat diet, Lanes 5: samples from rats on high fat diet. M= 100 bp DNA ladder. The upper 279 bp bands represent amplification of P53; the lower 211 bp band represents the internal control GAPDH (lane 1).

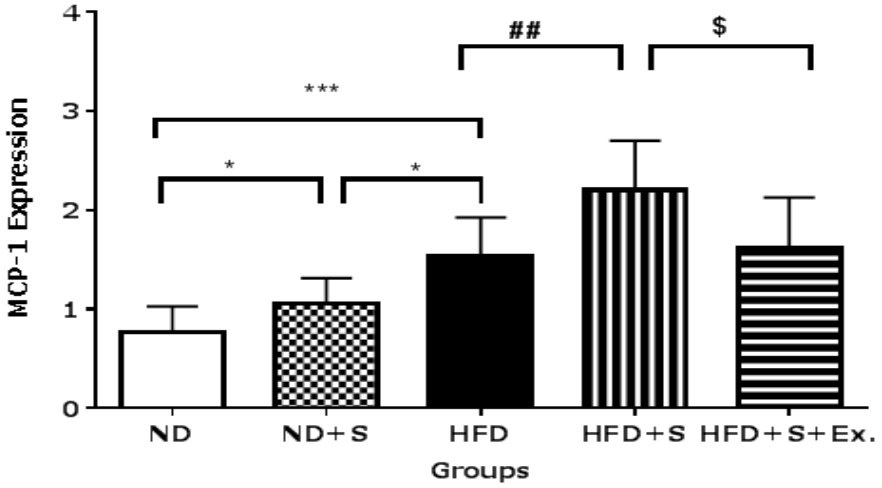


Fig.5.b. MCP-1 mRNA expression in visceral adipose tissue from normal diet group (ND), stressed normal diet group (ND-S), high fat diet (HFD), stressed high fat diet group (HFD-S), and stressed high fat diet with exercise group (HFD-S-E). Each group = 8 rats. All results are expressed as mean ± SD. P*, $p^{\$}$ <0.05, $p^{\#\#}$<0.01, p***<0.001 (one way ANOVA).

5. EFFECT ON GENES OF APOPTOSIS:

A. p53 gene expression in adipose tissue:

Images in (Fig. 6.a) show expression analysis of p53 mRNA from visceral adipose tissue extracted from different groups. There was significant increase in the expression of this proapoptotic gene due to HFD and chronic stress (Fig. 6). It was increased in stressed normal diet when compared to ND ($p<0.05$) and in non-stressed HFD group as compared to ND group ($p=0.0005$; see fig.6.b). Also, a significant difference in its expression was observed between stressed HFD group and non-stressed HFD ($p=0.0005$). On the other hand, its expression was lesser in stressed HFD with exercise group than in stressed HFD group ($p=0.0$; Fig.6.b).

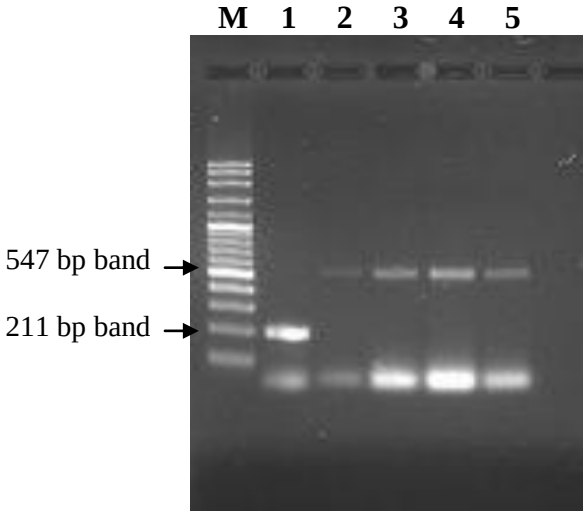


Fig.6.a. Expression analysis of p53 mRNA from visceral adipose tissues by RT-PCR. Lanes 2: samples from rats on normal diet, Lanes 3: samples from stressed rats on normal diet; Lanes 4: samples from stressed rats on high fat diet, Lanes 5: samples from rats on high fat diet M= 100 bp DNA ladder. The upper 547 bp bands represent amplification of P53; the lower 211 bp band represents the internal control GAPDH (lane 1).

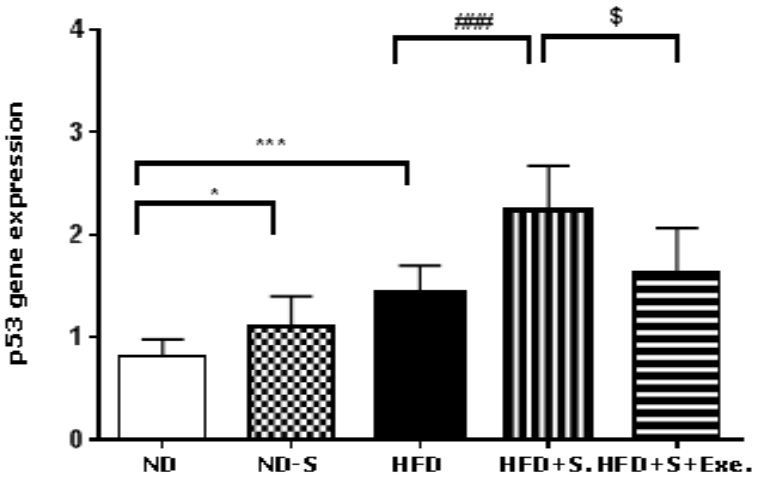


Fig. 6.b. p53 mRNA expression in visceral adipose tissue from normal diet fed rats (ND), stressed normal diet group (ND-S), high fat diet group (HFD), stressed high fat diet (HFD-S), and from stressed high fat diet with exercise (HFD-S-E). Each group =8 rats. All results are expressed as mean ± SEM. P^{*}, $p^{\$}<0.05$, p^{***}, $p^{\#\#\#}<0.001$ (one way ANOVA).

B. Bcl-2 gene expression in visceral adipose tissue

Images in (Fig. 7.a) show expression analysis of BcL-2 mRNA from visceral adipose tissue extracted from different groups. There was no significant difference in its expression in ND groups and HFD group (Fig.7). On the other hand, its expression was greatly reduced in HFD-S group as compared to HFD ($p<0.0001$). Although its expression was relatively greater in Stressed HFD with exercise group than in HFD-S group ($p<0.05$) but its level was still significantly less as compared to HFD group ($p=0.0009$; see Fig.7.b).

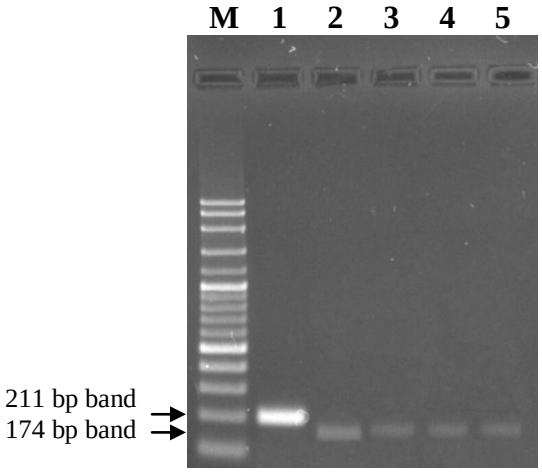


Fig.7.a. Expression analysis of Bcl-2 mRNA from visceral adipose tissues by RT-PCR. Lanes 2: samples from rats on normal diet, Lanes 3: samples from stressed rats on normal diet; Lanes 4: samples from stressed rats on high fat diet, Lanes 5: samples from rats on high fat diet M= 100 bp DNA ladder. The lower 174 bp bands represent amplification of Bcl-2; the upper 211 bp band represents the internal control GAPDH (lane 1).

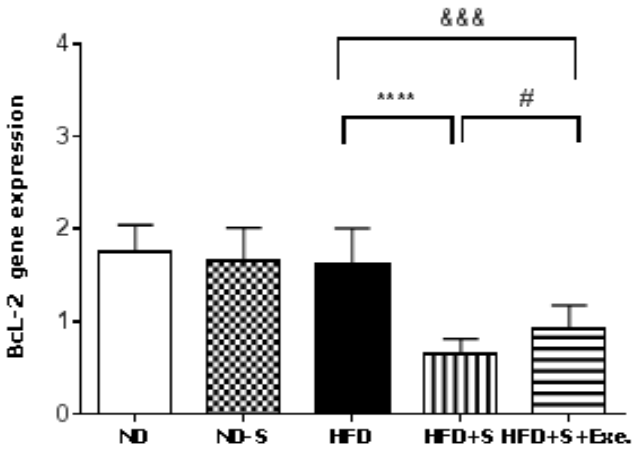


Fig.7.b. Bcl-2 mRNA expression in visceral adipose tissue from normal diet fed rats (ND), stressed normal diet group (ND-S), high fat diet group (HFD), stressed high fat diet group (HFD-S), and from stressed high fat diet with exercise group (HFD-S-E). Each group =8 rats. All results are expressed as mean ± SEM. $P^{\#}<0.05$, $p^{\&\&\&}<0.001$, $p^{****}<0.0001$ (one way ANOVA).

6. HISTOPATHOLOGICAL AND IMMUNOHISTOLOGICAL CHANGES:

Histopathological examination of heart and adipose tissue sections of rats exposed to stress alone or to stress with high fat diet showed abnormalities as compared to the ND fed rats. Heart tissue of ND fed rats showed normal striated myocytes with a single central ovoid nucleus with clear zones at the poles (Fig. 8). Heart tissue of the normal diet but exposed to stress showed increase in cell diameter and intercellular spaces with dilation and congestion in myocardial interstitial vasculature with increase in inflammatory cells, and there was also mild degeneration and hyperemia in myocardial cells (Fig. 9). Heart tissue of high fat diet fed rats showed hypertrophic cardiomyocytes containing fat droplets and areas of congestion and haemorrhage (Fig. 10). Heart tissue of high fat diet fed rats exposed to stress showed disarrayed cardiomyocyte with some degeneration and inflammatory cells infiltration (Fig. 11). Heart tissue of high fat diet fed rats exposed to stress and exercise showed areas of vaculated and infarct myocytes among the normal myocytes (Fig. 12).

The immune staining against p53 of the sections of the ND fed rat's heart tissue was negatively stained both in the cardiomyocytes, the fibroblasts and the endothelial cells lining the capillaries (Fig. 13). Heart tissue of the normal diet but exposed to stress showed positive reaction in the inflammatory cells in the wide intercellular spaces but still negative in the cardiomyocytes (Fig. 14). On the other hand, in the heart tissue of HFD fed rats either stressed alone or stressed and exercised, the p53 expression increased in the heart tissue sections was seen in the cardiomyocytes nucleus, cytoplasm and the nuclei of the fibroblasts in the interstitium (Figs. 15, 16, 17).

Moreover, p53 immune staining for the adipose tissue of the high fat diet fed group showed negative reaction in the ND group (Fig. 18) while in the other groups there was increased expression in both the cytoplasm and nuclei of the fat cells as compared to the control, while the expression was more intense in the stress exposed groups (Figs. 19, 20, 21, 22).

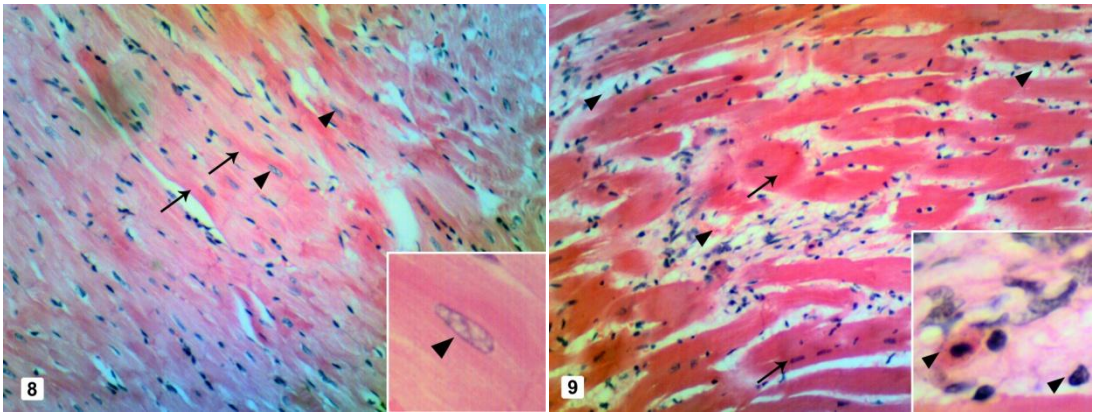


Fig. 8: A photomicrograph of a section of the heart of normal diet rat showing branching and anastomosing cardiac myocytes (arrows) with acidophilic sarcoplasm and central ovoid vesicular nuclei (arrowheads). Inset: High magnification showing a vesicular ovoid nucleus with clear zones at its pole (arrowhead).
Hx. & E., X100, Inset; X400

Fig. 9: A photomicrograph of a section of the heart of a normal diet with stress rat showing increase in the diameter of the cardiac myocytes with deeply stained acidophilic sarcoplasm and central ovoid vesicular nuclei (arrows). There is widening of the intercellular spaces with congested capillaries and inflammatory cells infiltration (arrowheads). Inset: High magnification showing inflammatory cells in the wide intercellular spaces (arrowheads).
Hx. & E., X100, Inset; X400

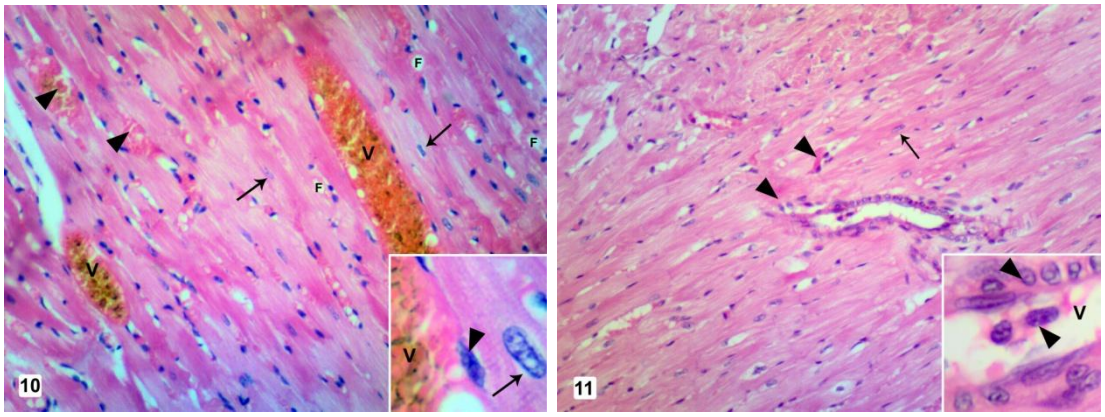


Fig. 10: A photomicrograph of a section of the heart in the high fat diet fed rats showing congested blood vessels (V) and areas of haemorrhages (arrowheads). Some cardiomyocytes are hypertrophic (arrows) containing fat cells (F). Inset: High magnification showing deformed cardiomyocyte nucleus (arrow) a spindle shaped nucleus of fibroblasts in the endomesium (arrowhead) close to a congested blood vessels (V).
Hx. & E., X100, Inset; X400

Fig. 11: A photomicrograph of a section of the heart in the high fat diet fed rats exposed to stress showing focal areas of necrotic fibers with vacuolatcd cytoplasm and stained pyknotic nuclei (arrowheads). Inset: High magnification showing inflammatory cells (arrowheads) within and surrounding blood vessel (V).
Hx. & E., X100, Inset; X400

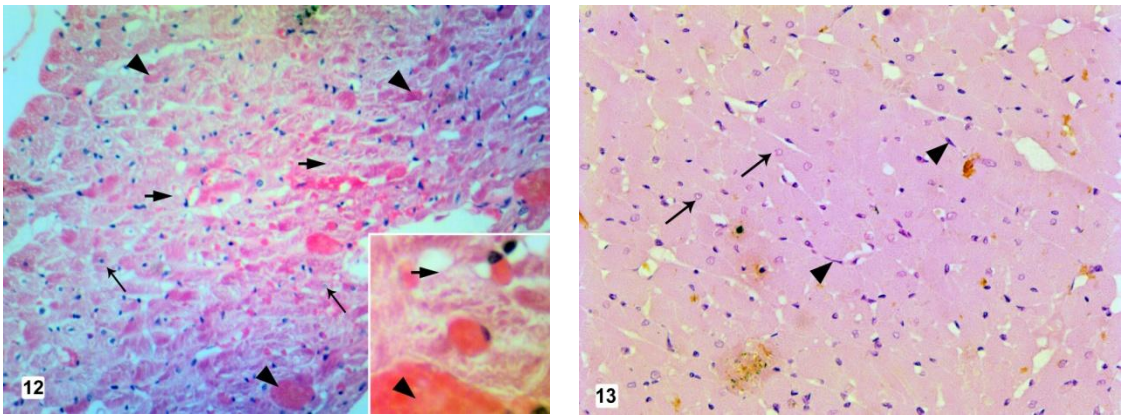


Fig. 12: A photomicrograph of a section of the heart in the high fat diet fed rats exposed to stress and exercise showing some infracted cardiomyocytes with hypereosinophilic cytoplasm (arrowheads) some fibers with lightly stained vacuolated cytoplasm (short arrows) and some normally appeared myocytes (arrows). Inset: High magnification showing infracted myocytes (arrowhead) and the vacuolated myocyte (short arrow).
Hx. & E., X100, Inset; X400

Fig. 13: A photomicrograph of a section of the heart of normal diet rat showing negative immune stain reaction for p53 in the cardiomyocytes (arrows) and the fibroblasts in the endomesium (arrowheads). P53 immunoperoxidase stain, X400

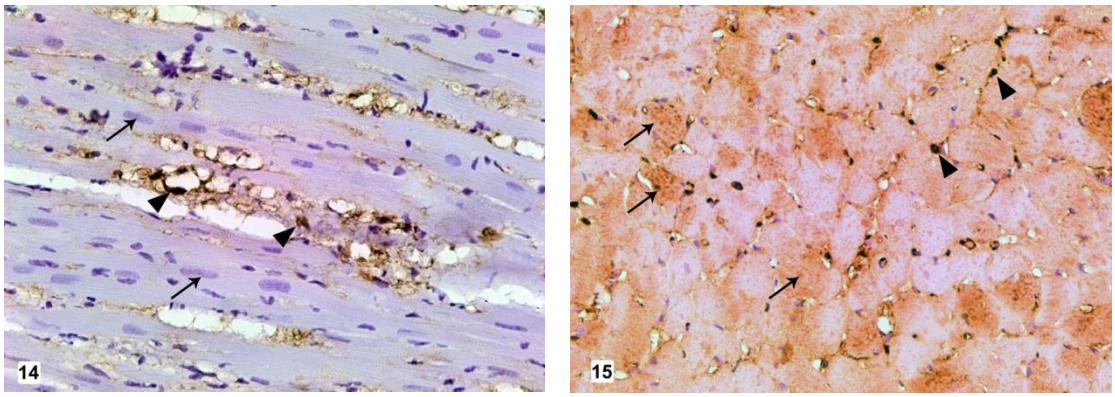
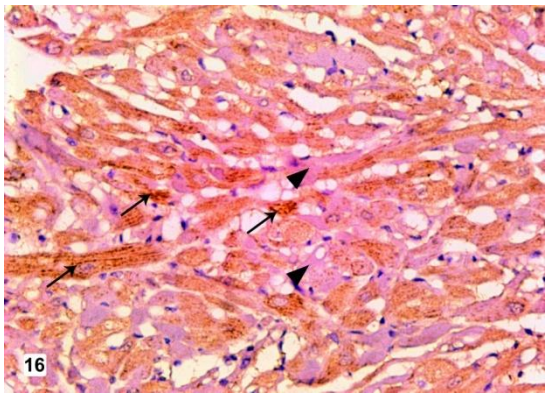


Fig. 14: A photomicrograph of a section of the heart of normal diet exposed to stress rat showing negative immune stain reaction for p53 in the cardiomyocytes (arrows) and positive reaction in the inflammatory cells in the intercellular spaces (arrowheads).
P53 immunoperoxidase stain, X400

Fig. 15: A photomicrograph of a section of the heart of the high fat diet fed rats showing positive immune stain reaction for p53 in the cardiomyocytes (arrows) and the fibroblasts in the endomesium (arrowheads).
P53 immunoperoxidase stain, X400



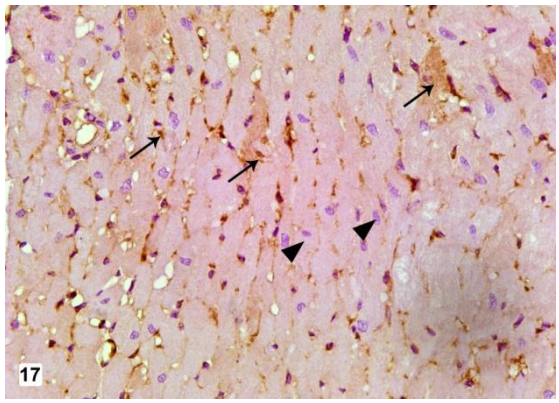
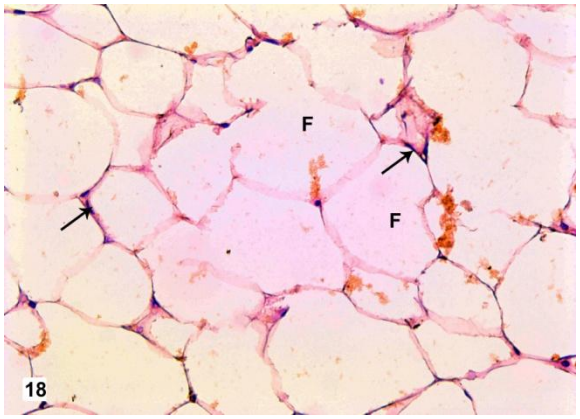


Fig. 16: A photomicrograph of a section of the heart in the high fat diet fed rats exposed to stress showing positive immune stain reaction for p53 in the cardiomyocytes (arrows) among necrotic negatively stained fibers (arrowheads).
P53 immunoperoxidase stain, X400

Fig. 17: A photomicrograph of a section of the heart in the high fat diet fed rats exposed to stress and exercise showing positive immune stain reaction for p53 in the cardiomyocytes and fibroblasts (arrows) among negatively stained fibers (arrowheads).
P53 immunoperoxidase stain, X400



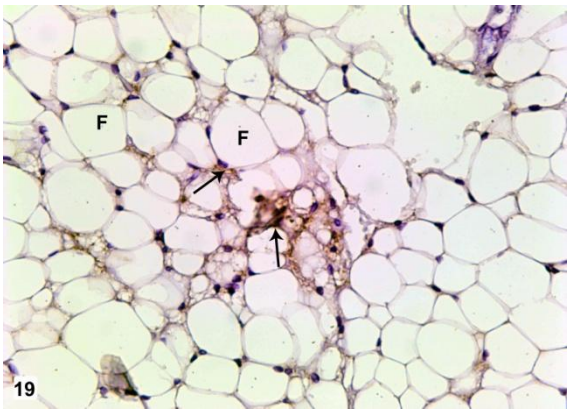


Fig. 18: A photomicrograph of a section of the adipose tissue of ***ND rat*** showing negative immune stain reaction for p53 in the nuclei (arrows) of the fat cells (F).
P53 immunoperoxidase stain, X400

Fig. 19: A photomicrograph of a section of the adipose tissue of ***normal diet with stress rat*** showing positive immune stain reaction for p53 in some nuclei (arrows) of the fat cells (F).
P53 immunoperoxidase stain, X400

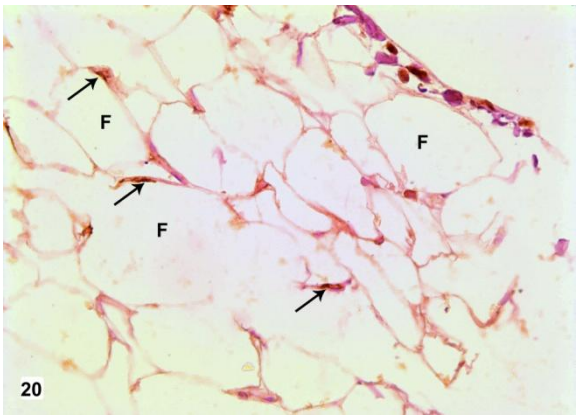
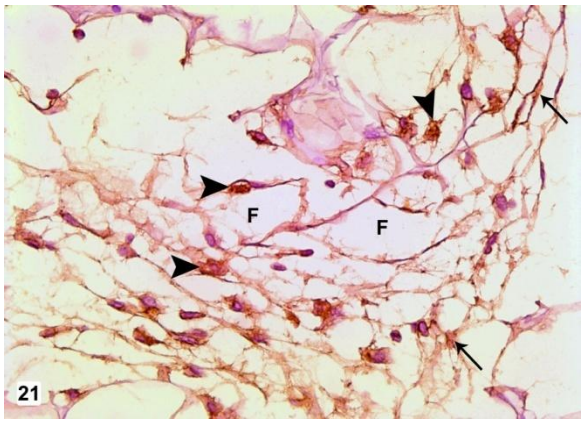


Fig. 20: A photomicrograph of a section of the adipose tissue of the ***high fat diet fed rats*** showing positive immune stain reaction for p53 in the nuclei (arrows) of the fat cells (F).
P53 immunoperoxidase stain, X400



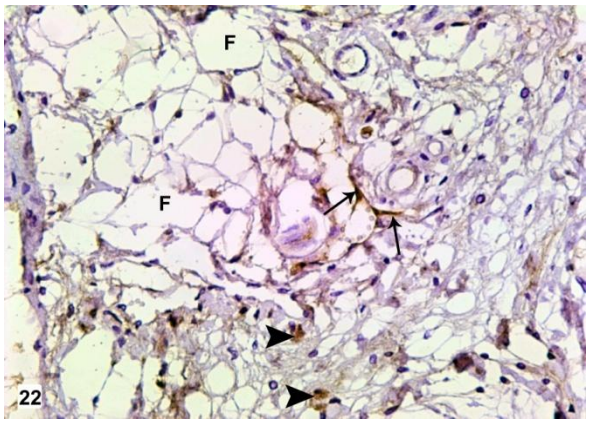


Fig. 21: A photomicrograph of a section of the adipose tissue in ***the high fat diet fed rats exposed to stress*** showing positive immune stain reaction for p53 in the nuclei (arrows) of the fat cells (F) which appeared collapsed. Note the positive reaction in the inflammatory cells (arrowheads). p53 immunoperoxidase stain, X400

Fig. 22: A photomicrograph of a section of the adipose tissue in ***the high fat diet fed rats exposed to stress and exercise*** showing positive immune stain reaction for p53 in the nuclei (arrows) of the fat cells (F). Note the positive reaction in the inflammatory cells (arrowheads). p53 immunoperoxidase stain, X400

Discussion

Inflammation and oxidative stress are thought to be involved in the association between obesity and CVD. Therefore, this study has tried to elucidate such link by assessing oxidative stress biomarkers in both adipose tissue and vascular tissue, namely cardiac muscle and aorta, in a rat model of obesity. Furthermore, assessment of apoptotic genes was done trying to explore the associated molecular changes. Moreover, we tried to investigate whether chronic exposure to different stressors, simulating what is happening daily, could aggravate the situation or not.

BW & Serum Corticosterone

High fat diet feeding for 12 weeks resulted in a significant increase in final body weight (Table 2). However, the combination of stress and high fat diet has not increased BW any further. Moreover, exposing normal diet fed rats to stress increased their BW slightly, suggesting that the stress does not play major role in body gain in rats. In the present experiment, serum corticosterone level, which was assessed as an index of stress, increased significantly in either HFD or stress alone. On the other hand, the results showed that the combination of HFD and stress has increased serum corticosterone level further in HFD fed group. An early study by Tannenbaum et al. (1997) has

reported similar effect on corticosterone levels in high-fat diet and chronic stress. By contrast, Paternain et al. (2011) have reported that serum corticosterone levels were decreased. Exercise seemed to reduce corticosterone levels to be comparable to that in HFD group, suggesting that exercise might alleviate the effect of stress but not the effect of high fat feeding.

Biochemical Parameters

Chronic consumption of HFD induced metabolic changes including increased glucose levels, insulin resistance and disturbed lipid profile as shown by the increase in TC, TG, LDL and the decreased HDL. Looking to the effect of stress alone on these parameters; it slightly disturbed blood glucose, TC & HDL in normal diet fed rats. However, when HFD was combined with stress, such disturbance was aggravated indicating the hazardous effect of combination of obesity and stress. On the other hand, exercise tended to improve such metabolic changes in comparison with stressed HFD group to be comparable with HFD group but was not that effective to bring it back to ND group level suggesting that the deleterious effect of obesity was more or less permanent as compared to the effect of stress. These results were in accordance with study of Touati et al. (2011).

Oxidative Stress Biomarkers (MDA, oxLDL, PG-Fα)

One main link that is thought to explain the deleterious effect of obesity is accumulation of products of oxidative stress with its hazardous consequences on both cellular and molecular levels. The present study has shown that the combination of HFD & stress has exerted greater effect on the production of oxidative stress biomarkers, MDA, oxLDL & PG-Fα. Specifically, OxLDL is known to be cytotoxic and may alter gene expression in arterial walls. Moreover, it is more readily taken up by macrophages that accumulate on arterial walls contributing to the development of atherosclerotic lesions (Cathcart et al., 1985). Interestingly, the histological examination of heart tissue sections of stressed ND group showed inflammatory cells infiltration in the widened intercellular spaces (Fig. 9). Further changes have been seen in heart tissues in HFD group where areas of haemorrhage and hypertrophied cardiomyocytes with fat cells exist, demonstrating the hazardous effect of HFD on vascular tissue . The combination of stress with HFD led to more extensive changes where focal areas of necrosis and vaculated cytoplasm were observed. Such finding supports previous reports that increased Ox-LDL production increases TG production and induces accumulation of fatty acids in adipocytes which stimulate accumulation of ceramide which leads to inflammation (Merkel et al., 2002). On the other hand, exercise has reduced the potentiating effect of stress with high fat feeding on ox-LDL & PG-F_{α} such that their level became comparable with that in HFD only. However MDA level was still higher even with exercise, suggesting a more deleterious effect of stress. In line with this, the histopathological changes observed in heart tissue in HFD group or stressed HFD rats were also seen in exercised stressed HFD rats. Whether the underlying reason for such incomplete improvement was insufficient episodes of exercise or its late start (4 weeks after start of stress & HFD) or the deleterious effect of combination of HFD and stress that interfered with improving by exercise is a question to be investigated further.

MCP-1 expression:

The present experiments have shown that MCP-1 expression is almost doubled in retroperitoneal adipose tissue in HFD group compared to ND group. Adipocytes have been recognized as an important source of MCP-1, and consequently with high fat diet where there is an expansion in both number and size of adipocytes, it would be logic to expect an increase in MCP-1expression. Higa et al. (2011) have reported similar results of high plasma MCP-1 in obese mice in comparison to lean controls. Earlier studies in human have shown that obesity is associated with higher MCP-1 expression (Catalan et al., 2007; Huber et al., 2008) indicating its role in obesity-induced chronic inflammation**.** The present study has added that MCP-1 expression is also enhanced in heart and aorta in correlation with that in adipose tissue suggesting its role in the link between HFD and initiation of CVD. Looking to chronic stress-induced change in MCP-1 protein expression, the exposure to stressors alone has significantly increased MCP-1 expression in adipose tissue however the HFD-induced increase in its expression was still greater when compared to stressed ND group. Indeed, few studies have investigated the effect of stress on MCP-1 expression. In their work on apoE−/− mice, Gu et al. (2009) reported an enhanced expression level of MCP-1 after the exposure to chronic-mild stress. Similarly, an increase in plasma MCP-1 was found in women having prolonged psychological stress (Asberg M, et al. 2009). By contrast, Paternain et al. (2011) has shown that chronic stress increased MCP-1 expression in retroperitoneal adipose tissue in control rats not in obese rats, suggesting that high fat diet could protect against stress-induced inflammatory response. However, the present work showed that a greater increase in mRNA MCP-1 expression was noticed when HFD is associated with stress, indicating the deleterious effect of such combination (Fig. 5.b). Although exercise has improved such deleterious effect on MCP-1 expression, but its level did not reach that of the control level.

Obesity, inflammation & apoptosis:

This study demonstrated that p53 expression is increased in adipose tissue from obese stressed rats and also stressed exercised obese rats. In line with this, sections of adipose tissue from HFD fed rats show positive immune reaction for p53 in nuclei of fat cells and such reaction is also observed in stressed HFD fed rats and even in exercised stressed HFD fed rats. More interestingly, p53 expression is increased in stressed ND fed rats and a positive p53 immune reaction is also observed in adipose tissue from stressed ND fed rats but is limited to some nuclei without involvement of inflammatory cells. Such finding highlights the effect of exposure to chronic stress per se on this apoptotic gene. Moreover, the p53 immune reaction in sections of heart tissue from different experimental groups is following the same pattern as the adipose tissue, indicating the close link between changes in adipose tissue and vascular tissue. Indeed, a relationship between adipose tissue inflammation and apoptosis was proposed, and the mechanism of p53 induction could be explained by various stresses that affect adipocytes as DNA damage, oxidative stress or hypoxia. The enlargement of cell volume might cause intracellular hypoxia in the expanding adipose tissue bed with release of TNF_α by adipocytes that could activate p53 (Keuper et al., 2011). Furthermore, the observed increase in blood glucose level tended to result in oxidative burden (Kaneto et al., 2001). Thus adipocytes are placed under stressful conditions with p53 activation. On the other hand, the present results showed insulin resistance in obese stressed rats. This could be due to attenuation of insulin signaling via phosphatidylinositol 3-kinase and Akt pathway and it is reported that this pathway plays an important role in the regulation of p53 by enhancement of ubiquitination and degradation of p53 (Ogawara et al., 2002). Hence, it is possible that loss of insulin suppression of p53 might be involved in this elevation of p53.

In this work, mRNA expression of Bcl-2 showed no difference between HFD fed rats and ND fed rats. However, its level was significantly decreased in stressed rats receiving high fat diet compared to HFD fed rats only, suggesting that the expression of this antiapoptotic gene was dramatically affected by chronic stress. As observed, exercise manages to improve its expression but its level was still significantly low. The demonstrated changes of decreased antiapoptotic (Bcl-2) mRNA expression were paralleled by an increase in mRNA expression of proapoptotic (p53) and MCP-1 expression as an inflammatory cytokines and macrophage infiltration marker in adipose tissues. This Bcl-2 antiapoptotic protein is localized to mitochondria, endoplasmic reticulum, and the nuclear envelope and interferes with the activation of caspases by preventing the release of cytochrome c (Alkhouri et al., 2010). Tinahones et al. (2013) have found a significant negative association between Bcl-2 mRNA levels and body mass index, suggesting a role for this antiapoptotic protein in the regulation of adipose tissue homeostasis. The relationship between the apoptotic pathway (proapoptotic caspases and antiapoptotic Bcl-2) and the proinflammatory condition of adipose tissue may underlie the morbidity of obese subjects (Guilherme, 2008). An interesting finding in the present experiments is that the expression of apoptotic p53 and the antiapoptotic Bcl-2 was greatly affected by chronic exposure to stress, especially Bcl-2, suggesting that the high fat diet potentiated the effect of stress. Indeed, this was in accordance with study of Sumis et al., (2013) where p53 upregulation was most apparent in HF fed, socially-isolated mice, but also seen in control diet fed mice exposed to social isolation stress.

In conclusion, the present experiment showed that obesity was associated with low grade inflammatory state, insulin resistance, and disturbed balance between proapoptotoic and antiapoptotic genes. The infiltration of adipose tissue with inflammatory cells with over production of oxidative stress products seems to play a crucial role in obesity-induced histopathological changes in adipose tissue as well as cardiovascular tissues. HFD and chronic stressors seem to potentiate the hazardous effect of each other.

References

1. Adams JM, Cory S (1998). The Bcl-2 protein family: arbiters of cell survival. Science; 281:1322–1326.
2. Alkhouri N, Gornicka A, Berk MP, et al (2010). Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis. J Biol Chem 285:3428–3438.
3. Allain CC, Poon LS, Chan CS, Richmond W & Fu PC. (1974). Enzymatic determination of total serum cholesterol. Clin Chem 20(4): 470-475.
4. Asberg M, et al. (2009). Novel biochemical markers of psychosocial stress in women. PLoS ONE; 4(1):e3590.
5. Badr KF, Abi-Antoun TE (2005). Isoprostane and the kidney. Antioxidant redox signal 7:236-43.
6. Batheja, N., Suriawinata, A., Saxena, R., etal. (2000). Expression of p53 and PCNA in cholangiocarcinoma and primary sclerosing cholangitis. Mod Pathol. 13 (12) 1265:8.

7. Burstein, M., Selvenick, H.R., Morfin, R., (1970). Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J. Lipid Res. 11, 583–595.

8. Catalan V, Gómez-Ambrosi J, Ramirez B, Rotellar F, Pastor C, Silva C, et al. Proinflammatory cytokines in obesity: impact of type 2 diabetes mellitus and gastric bypass. Obes Surg. 2007; 17:1464–74.

9. Cathcart, M. **K.,** D. W. Morel, and G. M. Chisolm. 1985. Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J Leukocyte Biol.* **38:** 341-350

10. Chandra J, Zhivotovsky B, Zaitsev S, Juntti-Berggren L, Berggren PO, Orrenius
Cohen, M. B. (2000). *Oncogene* **19,** 1959–1968

11. Curat CA, Miranville A, Sengenes C, et al (2004). From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes 53:1285–92.

12. Dallman MF, et al (2003). Chronic stress and obesity: a new view of "comfort food.". Proc. Natl. Acad. Sci. USA 2003; 100:11696–11701)

13. Debora Estadella D, Oyama DL, Ana R. Daˆmaso, Ribeiro EB & Oller Do Nascimento CM. (2004). Effect of Palatable Hyperlipidic Diet on Lipid Metabolism of Sedentary and Exercised Rats. *Nutrition* 20:218 – 224.

14. Eckel RH, Barouch WW, Ershow AG. (2002). Report of the National Heart, Lung, and Blood Institute-National Institute of Diabetes and Digestive and Kidney Diseases Working Group on the pathophysiology of obesity-associated cardiovascular disease. *Circulation*. 105:2923–2928.

15. Furukawa S, Fujita T, Shimabukuro M, et al (2004). Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest.114:1752–61

16. Gross M, Steffes M, Jacobs DR Jr, et al (2005). Plasma F2- isoprostanes and coronary artery calcification: the CARDIA Study. Clin Chem 51:125–131.

17. Gu H, Tang C, Peng K, Sun H & Yang Y. (2009). Effects of chronic mild stress on the development of atherosclerosis and expression of toll-like receptor 4 signaling pathway in adolescent apolipoprotein E knockout mice. J Biomed Biotechnol: 613879.

18. Guilherme A, Virbasius JV, Puri V, Czech MP. (2008). Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 9:367–377.

19. Guo Z, Van Remmen H, Yang H, et al (2001). Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxidized LDL-induced apoptosis in mouse aortic cells. Arterioscler Thromb Vasc Biol. 21:1131–8.

20. Higa JK, Liu W, Berry MJ, Panee J (2011). Supplement of bamboo extract lowers serum monocyte chemoattractant protein-1 concentration in mice fed a high fat diet. British Journal of Nutrition. 2011; 106:1810–3.

21. Huber J, Kiefer FW, Zeyda M, Ludvik B, Silberhumer GR, Prager G, et al. (2008). CC chemokine and CC chemokine receptor profiles in visceral and subcutaneous adipose tissue are altered in human obesity. J Clin Endocrinol Metab. 93:3215–21.

22. Hwang DL, Barsenghian G & Lev-Ran A (1985). Determination of free insulin in antibody containing sera: comparison of polyethylene glycol and staphylococcous aureus cells. Horm.Metab.Res., 17:595-597.

23. Kaneto, H., Xu, G., Song, K. H., Suzuma, K., Bonner-Weir, S., Sharma, A., & Weir, G. C. (2001). Activation of the hexosamine pathway leads deterioration of pancreatic beta-cell function through the induction of oxidative stress. *J. Biol. Chem.* 276**,** 31099–31104

24. Keuper M, Bl€uher M, Schön MR, et al. (2011). An inflammatory micro-environment promotes human adipocyte apoptosis. Mol Cell Endocrinol 339:105–113

25. Kuo L, Czarnecka M, Kitlinska JB, Tilan JU, Kvetňanskýb R, and Zofia Zukowska Z (2008). Chronic stress, Combined with a High-Fat/High-Sugar Diet, Shifts Sympathetic Signaling toward Neuropeptide Y and Leads to Obesity and the Metabolic Syndrome. Ann N Y Acad Sci. 1148: 232–237

26. Martinovic I, Abegunewardene N, Seul M, et al (2005). Elevated monocyte chemoattractant protein-1 serum levels in patients at risk for coronary artery disease. Circ J. 69: 1484–9.

27. Merkel M, Heeren J, Dudeck W, et al (2002). Inactive lipoprotein lipase (LPL) alone increases selective cholesterol ester uptake *in vivo*, whereas in the presence of active LPL it also increases triglyceride hydrolysis and whole particle lipoprotein uptake. J Biol Chem 277(9):7405–7411.

28. Michel C, et al. (2005). Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. Horm. Behav. 48:172–179.

29. Michel, C., Duclos, M., Cabanac, M., Richard, D. (2005) Chronic stress reduces body fat content in both obesity-prone and obesity-resistant strains of mice. *Horm Behav*. 48: 172–179.

30. Ogawara, Y., Kishishita, S., Obata, T., Isazawa, Y., Suzuki, T., Tanaka, K., Masuyama, N. & Gotoh, Y. (2002). Phosphorylation of AKT: a Mutational Analysis. *J. Biol. Chem.* 277, 21843–21850.

31. Ohakawa H, Oshishi N, Yagi K (1979). Assay For Lipid Peroxidation In Animal Tissue by Thiobarbituric Acid Reaction. *Anal. Biochem.*75: 351-358.

32. Paternain L., García-Diaz D.F., Milagro F.I., González-Muniesa P., Martinez J.A., & Campión J (2011). Regulation by chronic-mild stress of glucocorticoids, monocyte chemoattractant protein-1 and adiposity in rats fed on a high-fat diet. Physiology & Behavior 103; 173–180.

33. Pickavance LC, Brand CL, Wassermann K & Wilding JP (2005). The dual PPARalpha/ gamma agonist, ragaglitazar, improves insulin sensitivity and metabolic profile equally with pioglitazone in diabetic and dietary obese ZDF rats. Br J Pharmacol 144(3):308–16.

34. Rokhlin, O. W., Gudkov, A. V., Kwek, S., Glover, R. A., Gewies, A. S (2001). Role of apoptosis in pancreatic beta-cell death in diabetes. Diabetes; 50 (Suppl.1):S44–S47.

35. Sumis A, Andrade FO, Hilakivi-Clarke LA. (2013). Social isolation stress promotes obesity and mammary tumorigenesis and increases both Mdm2 and p53 expression and autophagy in the mammary gland. Cancer Res 73 (19 Suppl).

36. Srinivasan K, Viswanad B, Asrat L, Kaul CL, Ramarao P. (2005). Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening. Pharmacol Res 52(4):313-20.

37. Tannenbaum BM, Brindley DN, Tannenbaum GS, Dallman MF, McArthur MD & Meaney MJ (1997). High-fat feeding alters both basal and stress-induced hypothalamic–pituitary–adrenal activity in the rat. Am J Physiol 273(6 Pt 1):E1168–77.

38. Tinahones FJ, Araguez LC, Murri M, Olivera WO et al. (2013) Caspase Induction and BCL2 Inhibition in Human Adipose Tissue, A potential relationship with insulin signaling alteration Diabetes Care 36:513–521.

39. Touati S, Meziri F, Devaux S, Berthelot A, Touyz RM, Laurant P. (2011). Exercise reverses metabolic syndrome in high-fat diet induced obese rats. Med Sci Sports Exerc. 43(3):398-407.

40. Trinder P. 1969. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol 22: 158-161.

41. Werner M, Gabrielson DG, Eastman J (1981). Ultra-microdetermination of serum triglycerides by bioluminescent assay. Clin Chem. Feb; 27(2):268-71.

42. Zorn-Pauly K, Schaffer P, Pelzmann B, et al (2005). Oxidized LDL induces ventricular myocyte damage and abnormal electrical activity—role of lipid hydroperoxides. Cardiovasc Res. 66:74–83.