Nuclear Factor- κB (NF-κB) Expression in High Fat Diet-Induced Obesity and Insulin Resistance in Rats

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Abstract: Introduction: Obesity is a metabolic disease associated with insulin resistance and release of multiple cytokines as IL-6. Nuclear factor – kappaB (NF-κB) is accused to be a central mediator of inflammatory and stress responses occurring in obesity. **Aim of the work:** The aim of this work was to induce a model of obesity and insulin resistance in rates that simulate human obesity. Also to assess the level of IL-6 and the expression of NF-κB in liver and muscles of these rats and the effect of exercise on their levels. **Materials And Methods:** The study was carried on 21 male Wister rats divided into :- **group1**(7rats) the control group fed on diet derived from fat for 3 months. **Group 2** (14 rats) fed on high fat diet for the same period of time, after one month group 2 were subdivided equally into **group2A** the resting group and **group2B** practicing swimming exercise for the rest two months. At the end of the three months blood samples were taken from all rats for estimation of levels of glucose and insulin to estimate insulin resistance (HOMA), they were sacrificed and samples of their livers and muscles for estimation of levels of IL-6 by ELIZA and expression of NF- kB by western blotting. **Results:** After one month there was a significant increase in weight in group2 rats than the control,the weight gain continued at the end of the experiment with no effect of exercise. Insulin resistance was significantly increased with increasing weight, and decreased by exercise. As regard level of liver IL-6 , there was a significant increase in the exercising group2B than the control and the non-exercising group2A,while muscle IL-6 as well as liver likelihood ratio of NF- k B showed significant increase in both groups 2A and B than the control with no exercise effect. Activity of muscle NF- kB showed a significant increase in groups2A and B than the control, also in group2B than group 2A.**Conclusion:** High fat diet can lead to weight gain and obesity that is associated with increasing insulin resistance which can be improved by exercise. Obesity may be accompanied by release of NF-kB that can increase IL-6. Effect of exercise on the studied parameters is contradicting,

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Key Words: High fat diet, Insulin resistance, IL-6, NF- kB and Exercise.

1. Introduction

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex that controls the transcription of DNA. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens.[1-4] NF-κB plays a key role in regulating the immune response to infection . It has also been implicated in processes of synaptic plasticity and memory. [5-7]

In quiescent cells, NF-kappa B is located in the cytoplasm in an inactive form, bound to an inhibitor molecule called I kappa B. Stimulation of cells through a variety of mechanisms triggers a cascade of signaling events that ultimately results in the degradation of I kappa B by the proteasome. This degradation releases active NF-κB, which then translocates into the nucleus, where it binds to specific DNA sequences on its target genes, initiating the transcription of gene products including various cytokines (eg, interleukin [IL]-1, IL-6, IL-8, tumor necrosis factor), angiogenesis factors (eg, vascular endothelial growth factor), cell adhesion molecules (eg, intercellular adhesion molecule 1 and vascular cellular adhesion molecule 1), enzymes (eg, cyclooxygenase 2, nitric oxide synthase), and antiapoptotic factors.[8]

Obesity is a disease affecting increasing numbers of global populations, It has been demonstrated that increased fat mass is associated with increased macrophage infiltration, increased release of cytokines, adipokines and free-fatty acids from adipocytes and/or activated macrophages, and local insulin resistance .A central mediator of inflammatory and stress responses is the NF-κB family of transcription factors. [9-11]

Although a number of studies have explored the effect of obesity on inflammatory mediators, surprisingly few studies have directly compared activation of NF-κB itself in obese individuals with lean controls. The dynamic regulation of NF-κB activity during weight gain is thus unknown, and it is not known whether increased NF-κB signaling is presented before, simultaneously, or after metabolic parameters are affected. More specifically, the time course of inflammation induced in parallel with obesity on an high fat diet (HFD) has not been elucidated, including the organs involved, nor the dynamics of inflammation development.[12]

IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation,

hematopoiesis, inflammation and oncogenesis. Its activities are shared by IL-6-related cytokines such as leukemia inhibitory factor (LIF), cilliary neurotrophic factor (CNTF) and oncostatin M .[13]

IL-6 exerts its biological effects through a receptor complex composed of the IL-6 binding subunit gp80 and dimer of the signal-transducing receptor subunit gp130 .[14]

IL-6 acts as both a pro-inflammatory and anti-inflammatory cytokine. IL-6 is also a "myokine," a cytokine produced from muscle, and is elevated in response to muscle contraction.[15]

Adipose tissue is the source of a wide variety of molecules involved in the regulation of energy output and carbohydrate metabolism. Among these, proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, in particular, appear to play a role in modulating insulin sensitivity in peripheral tissues and have been associated with the development of insulin resistance in adults.[16] Furthermore, glucose induces oxidative stress and increased NF-κB binding, and it also increases the transcription of NF-κB –dependent proinflammatory genes (TNF-α and IL-6) .[17,18]

Aim of the work

The aim of this work is to induce a model of obesity and insulin resistance in rates by high fat diet to simulate human obesity. Also to assess the level of IL-6 and the expression of NF-κB in liver and muscles of these animal models and the effect of exercise on their levels.

2. Materials and Methods:

An approval of the ethical committee in faculty of medicine was obtained. A 21 male Wister rats (100-200) g body weight were fed as follows:

Group 1 :Control diet (5% of energy derived from fat, 18% from proteins and 77% from carbohydrates ; 3.3 Kcal/g)[19]

Group 2: High fat diet (HFD) (58% derived from fat, 18% proteins and 24% from carbohydrates ; 5.6K cal/g) . The amount of lipid was provided mainly by saturated fat as butter or dairy products also polyunsaturated fat as corn oil or sunflower oil.[19]

After one month, the rat group on HFD will be divided into:

Group2A: HFD sedentary group that continue on the same diet without exercise for additional 2 months.

Group 2B: HFD exercise trained. The exercise protocol consisted of swimming for 1h/day , 5 days/ week for additional 2 months. This protocol represents a moderate aerobic exercise training in rats.[20]

At the end of experiment rats were sacrificed , plasma samples were taken and liver and skeletal muscle were obtained for estimation of the following :

Fasting plasma insulin and glucose levels for calculation of IR according to **Matthews et al**[21] homeostasis model assessment of insulin resistance (HOMA-IR) using the formula:

lR = fasting insulin x fasting glucose / 22.5

Determination of IL-6 in liver & muscles by ELISA kit (Ray Biotech, Inc.) according to the manufacturer's instructions. [22]

Determination of Nuclear factor kB (NF-kB) expression by western blotting in liver and muscle: [23] For western blotting, equivalent concentrations of protein were separated by 10% SDS–PAGE and transferred onto nitrocellulose filter. The filters were then incubated in blocking solution for 2 hrs at room temperature. The filters were reacted with the anti-NF-kB antibody (R&D systems) at a dilution of 1:1000 for 2 hrs. Then the blot was washed three times (5 minutes each) with 1X PBST (0.05% Tween in phosphate buffered saline PBS) and then washed for 10 min with Tris buffered saline (TBS) with shaking. Filters were then incubated with horseradish peroxidase-conjugated secondary antibody (R&D systems) of 1:1000 for 1 h. After the secondary incubation the membrane was washed 3 times (5min each) with TBST (TBS with 0.05%Tween) and then washed again in TBS with shaking. 3,3′-Diaminobenzidine (DAB) substrate solution was prepared, then 30 µl hydrogen peroxide were added. After developing the color of the blot, the reaction was stopped after appearance of the expected bands by pouring out the substrate and rinsing with distilled water repeatedly. As an internal control, Beta Actin antibody (Affinity-purified Sheep Anti-human/mouse/rat Actin Antibody from R&D systems) was used as control.

Statistical Analysis:

Data were collected and analyzed using SPSS program version10. Qualitative data were described using number and percent. The values of the measured parameters were expressed as mean ± SD. Mann-Whitney and t-test were used to compare between two samples. Spearman Rho correlation coefficient was performed for evaluating correlation between the qualitative variables.

3. Results

Table 1 shows the weight gain among the studied groups after 1month , there is a significant increase in weight of rats on high fat diet (group2) than the control (group1)[Z=3.681 p <0.0001]. At the end of the experiment there is a significant increase in group 2A ,also in group 2B than the control group1, while there is no significant difference between group2A and

group 2B as shown respectively [Z=3.13, Z=3.134 p 0.001], [Z=0.771 p0.456].

Table 2 shows the values of insulin resistance and IL-6 in liver and muscle of the studied groups, where there is significant increase of the value of insulin resistance of group 2A and group 2B than control group [Z=3.13 p0.001,Z= 2.047 p=0.038] , also there is significant decrease in group 2B than group 2A [Z=2.747, p= 0.004]. As regard levels of liver IL-6 there is insignificant difference between group 2A and the control [Z=0.064 p=1.0] while a significant difference is found between group2 Band the control [Z=2.236 p=0.026] also between groups 2B and 2A[Z=2.108 p=0.038] . MuscleIL-6 values shows a significant difference between group 2A also group2B and control group1[Z=2.108 p=0.038 , Z=2.492 p=0.011] while there is insignificant difference between groups2B and2 A[Z=1.725p=0.097].

Likelihood ratio(LR) of nuclear factor- κB (NF-κB) expression of the studied groups is shown in table3, where a significant increase in liver NF- κB in group 2A and group2B than the control [LR=6.363, 13.86 p=0.042,0.003] while there is no significant difference between the rats on high diet with exercise or without groups 2B and 2A[LR=5.04 p=0.169]. LR of muscle NF- κB shows significant increase in groups 2A and B than the control group1[LR=11.032,19.41 p=0.042,‹ 0.0001], Also between groups 2Band 2B[LR=11.03 p=0.004].

Table 4 represents a correlation between weight gain at different intervals and insulin resistance with the studied parameters among the studied groups. There is a positive correlation between weight gain after 1 month and muscle IL-6 , liver and muscle NF-κB [r=0.562,0.553,0.773p=0.008,0.009, ‹0.0001] while at the end of the experiment there is positive correlation between the weight gain and liver and muscle IL-6 [r=0.524, 0.644p=0.015,0.002] also between weight gain and liver and muscle NF- κB [r=.606,0.638 p=0.004,0.002]. while table 5 shows the correlation between IL-6 and NF- κB among the studied groups of rats, where there was a positive correlation between muscle NF- κB and muscle IL-6 and liver NF- κB[r = 0.66, 0.817 p = 0.001, <0.0001] respectively.

Table1: Weight gain at the end of 1st and 3nd months of study among different groups of rats.

Weight gain	Groups	Min-Max	Mean±SD	P	P1	P2	P3
1st month	Control	10-28	17.7±5.5	Z=3.681 (<0.0001)*			
	Rats on high fat diet	45-85	63.2±12.8				
3ndmonth	Control	4-30	21.7±8.5		Z=3.13 (0.001)*	Z=3.134 (0.001)*	Z=0.771 (0.456)
	Obese with no exercise	34-51	43.1±6.6				
	Obese with exercise	35-62	46.9±11.1				

P: Significance between control and rats on high fat diet P1: Significance between control and obese rats with no exercise
P2: Significance between control and obese rats with exercise P3: Significance between obese rats with and without exercise.
Z: Mann Whitney test *significant at P≤0.05

Table 2: Results of insulin resistance and IL-6 (pg/mg protein) among different groups of rats

Parameter	Groups	Min-Max	Mean±SD	P1	P2	P3
Insulin resistance	Control	1.4-2.3	1.7±0.4	**Z=3.13 (0.001)***	**Z=2.047 (0.038)***	**Z=2.747 (0.004)***
	Obese with no exercise	2.4-5.7	4.7±1.1			
	Obese with exercise	1.6-3.6	2.5±0.7			
Liver IL-6	Control	128.0-278.9	195.2±53.8	Z=0.064 (1.0)	**Z=2.236 (0.026)***	**Z=2.108 (0.038)***
	Obese with no exercise	109.5-349.8	203.5±81.5			
	Obese with exercise	104.8-366.6	321.6±96.5			
Muscle IL-6	Control	15.5-43.9	29.8±10.6	**Z=2.108 (0.038)***	**Z=2.492 (0.011)***	Z=1.725 (0.097)
	Obese with no exercise	27.4-72.2	51.9±17.8			
	Obese with exercise	25.2-86.2	67.6±21.5			

P1: Significance between control and obese rats with no exercise P2: Significance between control and obese rats with exercise
P3: Significance between obese rats with and without exercise.
Z: Mann Whitney test *significant at P≤0.05

Table 3: Likelihood ratio of NF-kB among different studied groups .

NF-kB	P1	P2	P3
Liver NF-kB	**LR=6.363 P=0.042***	**LR=13.86 P=0.003***	**LR=5.04 P=0.169**
Muscle NF-kB	**LR=11.032 P=0.004***	**LR=19.41 P<0.0001***	**LR=11.03 P=0.004***

P1: Significance between control and obese rats with no exercise
P2: Significance between control and obese rats with exercise
P3: Significance between obese rats with and without exercise.
LR: Likelihood ratio *significant at P≤0.05

Table (4): Correlation between weight gain at different timings and insulin resistance with markers studied among studied rats.

Markers	Weight gain 1st month		Weight gain 1st month		Insulin resistance	
	R	P	R	P	r	P
Liver IL-6	0.327	0.148	**0.524**	**0.015***	-0.071	0.76
Muscle IL-6	**0.562**	**0.008***	**0.644**	**0.002***	0.343	0.128
Liver NF-kB	**0.553**	**0.009***	**0.606**	**0.004***	0.255	0.264
Muscle NF-kB	**0.773**	**<0.0001***	**0.638**	**0.002***	0.338	0.134

r: Spearman Rho correlation *significant at P≤0.05

Table 5: Correlation between IL-6 and NF-kB markers among studied rats

Markers	Liver IL-6		Muscle IL-6		Liver NF-kB	
	R	P	R	P	R	P
Liver IL-6	----	----				
Muscle IL-6	0.347	0.124	----	----		
Liver NF-kB	0.409	0.065	0.336	0.137	----	---
Muscle NF-kB	0.352	0.118	**0.66**	**0.001***	**0.817**	**<0.0001***

r: Spearman Rho correlation *significant at P≤0.05

4. Discussion

Genetic and environmental factors play a role in the development of obesity. Diet is one of the main environmental factors that contribute to this disease. Human studies have shown that increased fat intake is associated with body weight gain which can lead to obesity and other related metabolic diseases. [24-26] Our data showed increased weight of rats fed on high saturated fat, this finding is in agreement with other researches who denotes that diets with 60 kcal% fat are commonly used to induce obesity in rodents since animals tend to gain more weight more quickly.[27,28] Also, it has been shown that high-fat diets used in laboratory animal research contain more saturated fat are quite capable of inducing obesity in susceptible strains in contrast to animals fed similar amounts of fat containing fish oil that did not gain as much weight as those fed diets with more saturated fat [29] and were more insulin sensitive .[30]

The results of this study clarified that insulin resistance increased with weight gain that can be improved by exercise , this can be explained by the finding that muscular exercise can increase insulin sensitivity by several mechanisms[31], including : enhancing both GLUT4-dependent glucose transport in skeletal muscle , increasing skeletal muscle vascularization, mitochondrial neobiogenesis, and eventually tissue mass , repartitioning intracellular fat, thereby improving its utilization and fat mass loss.[32-34] Adenosine monophosphate-activated kinase (AMPK) activity- a key regulator of energy-modulating pathways in skeletal muscle is acutely increased by muscle contraction , it has been termed an "energy sensor" that shift the cell's metabolic activities away from substrate storage toward oxidative substrate disposal to create ATP.[35] Several of the early studies observed significant improvements in glucose tolerance and insulin sensitivity in response to exercise training and corresponding reductions in body fat.[36] **Duncan et al.**[37] describes the effects of exercise without weight loss on insulin sensitivity and several markers of lipid metabolism in a group of 18 sedentary men and woman. The principal finding was that insulin sensitivity and plasma lipase activity increased without a corresponding change in BMI, waist circumference, or cardiorespitarory fitness. The authors concluded that modest amounts of exercise without weight loss positively affect markers of glucose and lipid

metabolism in previously sedentary adults. It is reasonable to suggest that the beneficial impact of daily exercise on insulin resistance would be magnified if associated with diminished body weight.[38]

In this study the results of estimating liver IL-6 were increased significantly in rats on high fat diet performing exercise (group 2B) than the control group. Also, there was a significant increase between group 2B and the resting rats (group 2A).These results may denote that exercise induces an inflammatory status that is accompanied by production of inflammatory cytokines, thus it may have a role in increasing IL-6 by the liver. **Febbraio et al.**[39] tested the hypothesis that the liver releases IL-6 during exercise in human subjects by measuring IL-6 across the hepatosplanchnic viscera. It was observed that rather than releasing IL-6, the liver actually eliminates this cytokine during exercise.

Muscle IL-6 levels were found to be increased in rats of groups 2A&B on high fat diet without and with exercise than the control group . These results may prove that obesity is an inflammatory condition that is responsible for high level of IL-6. Also exercise may be accompanied by muscle injury, and accumulation of cellular debris in the areas of tissue damage, triggers an inflammatory reaction characterized, in part, by the production of cytokines. It was found also that there was no significant difference between groups 2A&B. This finding may be due to the effects of exercise intensity, duration and mode. Also, it may be due to the duration passed between the end of exercise and taking the muscle biopsy. This is proved by a large increase in mRNA expression of MCP-1, IL-6, and IL-8 at 2 h post exercise, and modest increases at 4 h.[40] Although number of studies have demonstrated that working muscle produces IL-6, muscle biopsies obtained before and after exercise in human subjects and rats demonstrate very little IL-6 mRNA in resting muscle but a 100-fold increase in exercising skeletal muscle.[41-43]

Furthermore, several human studies have shown a marked increase in circulating IL-6 during prolonged exercise, mainly due to increased local production in the working skeletal muscle.[44-46] In rodents there is also an increase in IL-6 expression in working muscle and plasma IL-6 concentration during exercise .[43,47,48]

One study protocol used repeated sprint swimming trials showed increased markers of muscle inflammation (IL-6 and C-reactive protein). The significant increase in plasma IL-6 concentrations was observed in those subjects after the third sprint swimming as compared to in-water group. This may reflect a pronounced inflammation in the muscle and be a factor causing further muscle damage indicated by the increased levels of CK (creatine kinase) in the blood during the repeated sprint swimming bouts. [49]

The study showed a significant increase in liver NF-κB in groups 2A&B than the control group. This can be explained by the fact that high fat diet promoted inflammation and NF-κB activation . [50] **Fan et al.**[51] reported that NF-κB binding activity was higher in the rats fed HFD diet than that in the controls. Also it has been shown that consumption of a HFD enhanced NF-κB p65 subunit activation in rat liver. [52] It was reported that HFD causes twofold increase in hepatic NF-κB activity indicating that the NF-κB pathway in liver is activated by both genetic (hyperphagic) and diet-induced obesity.[53]

The present study doesn't show a significant difference between groups 2B and 2A which denotes that there is no effect of swimming exercise on liver NF-κB. This can be explained as liver is concerned with elimination of the cytokine rather than producing it.

The present study showed a significant increase in the level of muscle NF-κB both in groups 2A&2B than the control group , also between the exercising group 2B and the resting group 2A. These findings may denote that both obesity as well as exercise can induce inflammatory cytokines mediated by release of NF-κB. This finding is in agreement with a study which demonstrated that insulin-resistant subjects have increased NF-κB activity in muscle.[54] The stimulation of NF-κB by exercise seems counterintuitive, since the NF-κB pathway has been associated with insulin resistance, whereas physical activity typically improves insulin sensitivity.[54] Also , it was found that acute cycle exercise increased NF-κB activity in nondiabetic (lean and obese) subjects. Furthermore, the stimulatory effect of aerobic exercise on NF-κB activity observed in humans is in line with results from prior studies performed in rodents .[55-57] The reason for the discrepancy with the human study by **Durham et al.** [58] which showed a reduction in NF-κB activity, is likely related to the different modes of exercise utilized.

Our data showed a positive correlation between weight gain and the studied parameters specially at the end of the experiment. This finding can be explained by many studies that demonstrated that obesity is an inflammatory condition involving many components of the classical inflammatory response to pathogens and includes systemic increases in circulating inflammatory cytokines .Nonalcoholic fatty liver disease (NAFLD) is associated with an increase in M1/Th1 cytokines and quantitative increases in immune cells. There is also evidence of increased inflammatory cytokine production and increased inflammation in skeletal muscle in obesity.[59]

HFD was accompanied by a two fold increase in hepatic NF-κB activity.[53]Also , it was demonstrated that insulin-resistant subjects have increased NF-κB activity in muscle.[54] Muscle from obese and T2DM

subjects had increased basal IL-6 mRNA levels by about twofold .[40] IL-6 might also play an indirect deleterious role in NAFLD pathogenesis. In diet-induced obese mice, treatment with IL-6 antibodies improved sensitivity to insulin.[60] Liver steatosis activates IKK-β and NF-κB, which upregulates IL-6 production and secretion.[53]

In this study , there was a positive correlation between muscle NF-κB activity and muscle IL-6 as the data provide solid evidence that acute exercise in lean adults stimulates muscle NF-κB activity correlating with the transient release of proinflammatory cytokines such as IL-6 .[54]It was also recorded that specific κB binding sites on the promoter region of genes coding MCP-1, IL-6, and IL-8 were identified that make these inflammatory myokines as both key activators and downstream target genes of the NF-κB signaling pathway. [40]

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