



A Quick Model for the Induction of Metabolic Syndrome Markers in Rats

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Abstract

Obesity, dyslipidemia, and glucose utilization disorders are the main indicators in the diagnosis of metabolic syndrome. However, there are other markers of damage from oxidative stress, such as the LDL cholesterol (LDL-c) plasma concentration, protein oxidation, lipoperoxidation, and DNA damage. In this study, male Wistar rats (250-270 g) were used. The rats were distributed into eight groups as follows: 1) standard diet and purified water intragastrically (1ml/kg) for seven weeks (N); 2, 3, 4, 5, 6 and 7) hypercholesterolemic diet and 60% fructose for four days (4D); one (1W), two (2W); three (3W), four (4W), five (5W), six (6W) and 7 (7W) weeks respectively and 8) 60% fructose for four weeks (4WF). At the end of treatment, the concentrations of total cholesterol (TC), HDL cholesterol (HDL-c), triglycerides (TGs), and glucose, as well as the alkaline phosphatase activity (ALP), were determined. Moreover, the percentage of cells with DNA migration was determined with a comet assay, and a histopathological study of the hepatic samples was conducted.

The animal model in the current study showed metabolic syndrome (MS) in animals after four weeks with the following well defined markers: decreased HDL-c; increased total cholesterol, LDL-c, atherogenic index, weight gain, and liver weight; hypertension; and defined steatosis leading to an increase in plasma ALP and genotoxic liver damage.

Keywords: Metabolic syndrome; Hypolipidemic; Hypoglycemic; Rat; Hypercholesterolemic diet; Fructose; Wistar rat

Introduction

Clinical identification of MS is controversial because there is not one single definition that precisely describes the syndrome. However, the components of MS, such as hypertension, obesity [1,2], glucose utilization disorders [3], liver damage [4,5], and dyslipidemia [6,7], are well established.

Oxidative stress, which affects LDL circulation, induces oxidation of other proteins and causes DNA damage, which is among the factors that influence the development of these metabolic alterations [8].

The prevalence of MS and insulin resistance in a population varies depending on the definition being used as well as the ethnic group, gender, and age of the studied population. MS is considered as an entity that includes chronic degenerative diseases, such as cardiovascular risk factors, thus converting MS into a polygenic and multifactorial clinical situation [9,10] in which the prevalence is increasing in parallel with the incidence of diabetes mellitus type II (DMII), android obesity, and cardiovascular diseases associated with dietary habits and unhealthy lifestyles. These unhealthy life styles include diets rich in saturated fats, simple sugars, and fructose as well as tobacco use, alcoholism, and sedentarism.

Patients with MS have hyperlipidemia and hyperglycemia, which are important cardiovascular risk factors due to endothelial exposure to high oxidative stress. Individuals with high blood glucose concentrations maintain a directly proportional relationship to nitric oxide inactivation, platelet aggregation, superoxide anion production, and prothrombotic states [11-13].

The decision regarding the model to be used in an experiment is often multifactorial. It is ideal for a study to be conducted in different models as the current available models do not completely reflect the

complexity of human physiology and the findings cannot be easily extrapolated to the clinical setting.

There are different rodent models to study the MS, however, the majorities are focused on specific aspects of the different metabolic alterations observed in the MS. The objective of these models could be approached as: 1) models that explain the physiopathologic and biochemical mechanisms, 2) models in which some MS signs are induced, and 3) those designed to evaluate the therapeutic strategies to overcome MS [14-16].

Currently, some preclinical models of MS have been proposed that provide relevant information regarding the majority of its indicators, and the most widely used models are implemented in rodents [1,17] even though they are not reproducible and are expensive in some cases.

In this study, the use of a relatively economical and simple model for the development of MS indicators is proposed with the purpose of using this model in the future to evaluate the effect of agents that may prevent MS or may be used in the treatment of MS.

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Methods

Animals and treatment

Male Wistar rats (250-280 g) from the vivarium of the Autonomous Metropolitan University [Universidad Autónoma Metropolitana] were used. The animals were placed in cages with sterile sanitary beds and were maintained under controlled temperature and humidity conditions with 12h light/dark cycles. The rat weights were recorded, and the rats were randomly distributed into the study groups. The Laboratory Rodent Diet 5001 (PMI, Richmond, IN) was used as the standard diet, which was ground to prepare a hypercholesterolemic diet containing 1% USP cholesterol (Sigma-Aldrich, St Louis, MO, USA), 0.5% sodium cholate (Sigma-Aldrich, St Louis, MO, USA), 5% pure butter (Arla Foods a/s, Viby, DK), 30% sucrose (Valle Verde, Jalisco, MX), 10% casein (Fronterra LTD, Auckland, NZ), and 53.5% standard food mixture [18]. The standard food mixture and drinking water were administered *ad libitum* during the treatment period. The fructose was prepared at a concentration of 60% in purified water and was administered intragastrically once per day.

The animals were divided into eight groups with eight animals each as follows: 1) standard diet and purified water intragastrically (1 ml/kg) for seven weeks (N); 2) hypercholesterolemic diet and 60% fructose for four days (4D); 3) hypercholesterolemic diet and 60% fructose for one week (1W); 4) hypercholesterolemic diet and 60% fructose for two weeks (2W); 5) hypercholesterolemic diet and 60% fructose for three weeks (3W); 6) hypercholesterolemic diet and 60% fructose for four weeks (4W); 7) 60% fructose for four weeks (4WF); and 8) hypercholesterolemic diet and 60% fructose for seven weeks (7W).

At the end of each treatment, the weight of each animal was recorded, and blood samples were collected via retro-orbital sinus puncture when the animals were anesthetized with pentobarbital sodium via the intraperitoneal route. Blood samples were placed in tubes without anticoagulant, and they were later centrifuged at 13000×g for 15 min to obtain serum.

Livers and the epididymal fat of each animal were collected and weighed. The livers were then immersed in a phosphate regulating solution and dried. A portion of each liver was then placed in a formalin solution.

The procedures used followed the Ethics Code for working with animals of the National School of Biological Sciences according to the guidelines set by the Official Mexican Standard (NOM-062-ZOO-1999).

Serum analysis

In the serum, the concentrations of glucose, TC, HDL-c, and triglycerides TGs as well as alkaline phosphate activity (ALP) were quantified using reactive kits (RANDOX Laboratories Limited, Country Antrim, UK) for the Selectra II Vita Lab automatic equipment from the Wiener Lab. Moreover, the LDL-c was calculated by applying Friedewald's formula [19].

Comet assay

The alkaline electrophoresis comet assay was performed according to a previously described procedure [20,21]. Briefly, a layer of ABPF was placed on frosted slides (1%). ABPF (100 µl) was then mixed with 50 µl of blood, and a second layer was applied on the slide. Finally, a third layer of ABPF was added. The slides were immersed in a fresh lysing solution for 24 h at 4°C to expose the DNA. The solution was composed

of 2.5 M NaCl (J.T. Baker, Edo. Mex, MX), 100mM EDTA (Sigma, St Louis, MO, USA), 10 mM trizma base (pH 10), 1% triton X-100, and 10% DMSO (Sigma, St Louis, MO, USA). The slides were then immersed in an alkaline buffer (300 mM NaOH (J.T. Baker, Edo. Mex, MX) and 1 mM EDTA; pH>13) for 20min. Electrophoresis was conducted for 20 min at 25 V (0.66V/cm) and 30mA at the same pH. The slides were then washed with a neutralizing buffer (pH 7.5) for 5 min and placed in a humidity chamber for their reading. Finally, the slides were stained with 50 µl of Hoechst stain for 1 min. The nucleoids were examined at 40X with an epifluorescence microscope (Axiophot-1 Zeiss) connected to a digital camera (Cool SNAP Pro Media Cybernetics) with capture, processing, and image analysis software (Image Pro-Plus version 5.0). One hundred images of 100 nucleoids were captured per animal, and these images were measured length wise and divided by the width of the diameter of the head ($\text{index} = l/w$). The percentage of cells with DNA migration versus those without migration was determined.

Histopathological analysis

The liver cuts obtained from the microtome were stained with hematoxylin and eosin, and they were examined under a standard light microscope. The fat droplet quantities were evaluated in random fields at 400X with Image J morphometry software.

Statistical analysis

The results are expressed as means \pm standard deviation. The results were analyzed using a one-way ANOVA and Student-Newman-Keuls test using Sigma Stat software (version 2.03), which established a significant difference when $p < 0.05$.

Results and Discussion

In this study, Wistar rats and important characteristics were used to evaluate the manifestation of the following MS markers: obesity, hypertension, hyperglycemia, dyslipidemia, non-alcoholic fatty liver, increased ALP activity, and genetic material damage.

The results of the present study showed (Table 1) a significant increase in the indicators of LDL-c and TC with respect to the standard diet group (N) after the first week. These results are similar to those found in models where 60% fructose is administered to rats with a predisposition to the disease, such as Sprague Dawley rats [22,23]. With respect to TGs, there was an important increase reaching statistical significance at seven weeks. This increase was due to excessive consumption of carbohydrates in the diet, which created an increase in hepatic fatty acid synthesis and its incorporation into very low density lipoproteins (VLDLs) [24]. The increase in the TG and VLDL formation was related to the increase in LDL because half of the VLDLs in plasma were converted into LDL [25].

Although the decrease in HDL-c was not significant, it exhibited a tendency to decrease MS characteristics according to the WHO [13-18]. HDL had an important impact on the calculation of the atherogenic index because an important gradual increase in its value was observed, which constituted a cardiovascular disease risk factor [26].

The increase in glucose was significant until the end of the seventh week. Even though acutely administered fructose does not increase insulin concentrations, chronic exposure can indirectly create compensatory hyperinsulinemia [24]. This condition can be created through mechanisms involving a fructose transporter (GLUT5) that has a role in MS insulin resistance, which leads to an increase in the plasma glucose concentration [8].

Table 2 shows that the average weight of the animals increased week by week and it was significantly higher in the seventh week as compared to the N and 4WF groups. The many similarities between the metabolic state of obese rats and obese humans, such as hyperinsulinemia, insulin resistance, and development of diabetes, have to be taken into account. Unfortunately, the obesity characteristics in humans and in rats differ, and the differences primarily occur in the observed relative body fat distribution and functional characteristics of adipocytes [1]. The increased observed body weight could be primarily attributed to the weight of the constituent fat, which increased by 381.33% in relation to the control group (N), as well as the increase in the relative weight of the liver, which increased significantly in the fourth week and was 67.84% higher with respect to the control (N) group at seven weeks.

Fructose administration decreases the transcriptional activity of PPAR, which leads to a reduction in mitochondrial catabolism of fatty acids [23]. This alteration together with the increase in hepatic fatty acid synthesis caused by fructose increases the availability of fatty acids for their use in the synthesis of hepatic triglycerides. These hepatic triglycerides will then accumulate (steatosis) or be secreted into the blood stream and be transported by VLDLs [27].

ALP activity was significantly increased by the fourth day of administration of the hypercholesterolemic diet and 60% fructose, which reflected the increase in steatosis in the liver [28]. However, this activity was regulated during the experimental weeks without reaching values close to those of the control (N) group.

Increases in the accumulation of triglycerides in the liver are generally due to increases in non-esterified fatty acids in the peripheral adipose tissue or increased lipid synthesis in the liver [29]. The development (posterior or simultaneous) of steatohepatitis can cause

additional damage (such as oxidative stress or genetic predisposition), which leads to an inflammatory response and possible fibrosis [30]. The development of non-alcoholic steatohepatitis has been explained as a “double impact theory”, with the first impact being the accumulation of triglycerides in the liver and the second impact being additional damage, such as inflammation or oxidative stress. In the present study, liver fat accumulation occurred in the first week and was constant throughout the weeks as shown in the micrographs (Figure 1). In the group that received the hypercholesterolemic diet and 60% fructose for seven weeks, the loss of cytosol was clearly observed with the presence of white spaces produced by fat vacuoles, which deformed the hepatocytes, giving them a balloon shape. Moreover, the manifestation of inflammation was observed with the presence of polymorphonuclear cells, which are the main indicators of a non-alcoholic fatty liver [31].

The arterial pressure of the animals treated with the hypercholesterolemic diet significantly increased during the fourth week but significantly decreased during the seventh week. This variation may be explained by an increase in the production of catecholamines in the sympathetic nervous system associated with an increase in the concentration of plasma insulin. Additionally, the action of insulin increasing fluid reabsorption at the proximal tubule can also be considered as an explanation for this behavior [32]. The hypertension produced by the high consumption of fructose and salt is associated with increased expression of angiotensin II type 1 receptor in adipose tissue [33]. The present animal study indicated that angiotensin II stimulated fibrosis, caused insulin resistance, and stimulated pro-inflammatory cytokines (Figure 2).

Genotoxic compounds are compounds that act directly or indirectly on DNA and are caused by a clastogenic event. Genotoxic potential

Diet	Cholesterol (mmol/L)	HDLcholesterol (mmol/L)	LDL-cholesterol (mmol/L)	Triglycerides (mmol/L)	Glucose (mg/dL)	IA
N	16.14 ± 5.64	6.97 ± 1.45	8.84 ± 6.67	0.71 ± 0.13	96.42 ± 26.49	1.9
4D	21.28 ± 4.3	4.86 ± 0.37*	16.15 ± 4.46	0.5 ± 0.11	102.71 ± 21.46	4.4
1W	30.71 ± 9.89*	5.34 ± 2.35	25.04 ± 11.08*	0.729 ± 0.26	102.71 ± 7.47	5.9
2W	36 ± 6.58*	5.07 ± 1.74	30.62 ± 8.16*	0.643 ± 0.331	84.28 ± 7.80	8.9
3W	44.14 ± 14.66*	3.61 ± 1.10*	40.31 ± 15.37*	0.486 ± 0.17	90.28 ± 12.91	10.2
4W	73.14 ± 16.12*	3.52 ± 1.57*	70.07 ± 17.40*	0.613 ± 0.55	100.62 ± 13.06	28.8
4WF	19.71 ± 3.14*	9.15 ± 1.28	10.22 ± 3.53	0.757 ± 0.29	99.28 ± 12.12	2.4
7W	88.25 ± 24.33*	4.913 ± 2.50	82.834 ± 24.1*	1.125 ± 0.24*	124.8 ± 6.3*	22.7

*Significant differences with respect to the control group (N) according to a one-way ANOVA and Student-Newman-Keuls test. The following abbreviations are used: N, standard diet and purified water fed intragastrically (1ml/kg) for seven weeks; 4D, hypercholesterolemic and 60% fructose for four days; 1W, hypercholesterolemic diet and 60% fructose for one week; 2W, hypercholesterolemic diet and 60% fructose for two weeks; 3W, hypercholesterolemic diet and 60% fructose for three weeks; 4W, hypercholesterolemic diet and 60% fructose for four weeks; 4WF, 60% fructose for four weeks; and 7W, hypercholesterolemic diet and 60% fructose for seven weeks

Table 1: Lipid profile, plasma glucose, and atherogenic index in rats with different exposure times to a hypercholesterolemic diet and 60% fructose.

Diet	Final weight (g)	Relative weight of epididymal fat (%)	Relative weight of the liver (%)	ALP (U/L)	Steatosis (%)
N	395.71 ± 16.49	4.34 ± 2.24	1.47 ± 1.8	183 ± 71	0
4D	271 ± 9.84	1.43 ± 0.5	1.18 ± 1.32	579.42 ± 182.14*	0
1W	303.57 ± 32.74	2.30 ± 1.04	1.76 ± 2.96	466.28 ± 44.18*	12.25 ± 0.6*
2W	326.14 ± 21.38	2.46 ± 0.95	1.55 ± 2.81	417.43 ± 139.19*	15.21 ± 4.89*
3W	346.5 ± 18.79	1.98 ± 0.58	1.65 ± 1.5	371.28 ± 29.60*	20.75 ± 4.87*
4W	390.87 ± 29.41	2.99 ± 9.06	2.03 ± 3.01*	386 ± 88.48*	36.17 ± 5.95*
4WF	346.57 ± 40.84	3.62 ± 0.35	1.25 ± 1.87	197.71 ± 79.01	0
7W	481 ± 37.28*	16.55 ± 4.06*	2.47 ± 5.7*	381.25 ± 87.82*	43.68 ± 3.67*

*Significant differences with respect to the control group (N) according to a one-way ANOVA and Student-Newman-Keuls test. The following abbreviations are used: N, standard diet and purified water fed intragastrically (1ml/kg) for seven weeks; 4D, hypercholesterolemic and 60% fructose for four days; 1W, hypercholesterolemic diet and 60% fructose for one week; 2W, hypercholesterolemic diet and 60% fructose for two weeks; 3W, hypercholesterolemic diet and 60% fructose for three weeks; 4W, hypercholesterolemic diet and 60% fructose for four weeks; 4WF, 60% fructose for four weeks; and 7W, hypercholesterolemic diet and 60% fructose for seven weeks

Table 2: Animal weight, epididymal fat, relative weight of the liver, ALP activity, and steatosis in Wistar rats with different times of exposure to a hypercholesterolemic diet and 60% fructose.

is a primary risk factor for chronic or long-term effects, such as carcinogenic effects and reproductive toxicity. Genotoxic biomarkers, which may define a pre pathogenesis state and could be a starting point for the prevention of the disease, are to be identified. Several of the widely used genotoxic biomarker assays include sister chromatid exchange, micronuclei, chromosomal aberration, and comet assays. In general, sister chromatid exchange tests and comet assays are of great use in this evaluation due to their capability to detect chronic and acute damage, respectively, as well as for the rapidity with which they can be conducted and their potential use for the evaluation of any eukaryotic cell population [34].

The comet assay using blood samples from rats fed the hypercholesterolemic diet and 60% fructose for seven weeks showed a 5.8% increase in damage as compared to the 1.5% increase in damage for the control group (N), which indicated that MS caused damage at the blood level. Analyzing the liver values obtained using the comet assay indicated that 75.5% of each set of 100 cells were damaged (Table 3 and Figure 3), which suggested that the liver was the most affected. As a result of insulin resistance associated with DM, obesity, and hyperlipidemia, an accumulation of fatty acids (FAs) occurs in the liver and β -oxidation is increased, thereby altering ATP homeostasis. All of these changes cause oxidative stress, which has harmful effects on cells, such as increased lipoperoxidation of cellular membranes, cellular degeneration, necrosis, apoptosis, expression of pro-inflammatory cytokines, and activation of stellate cells in the liver, giving rise to

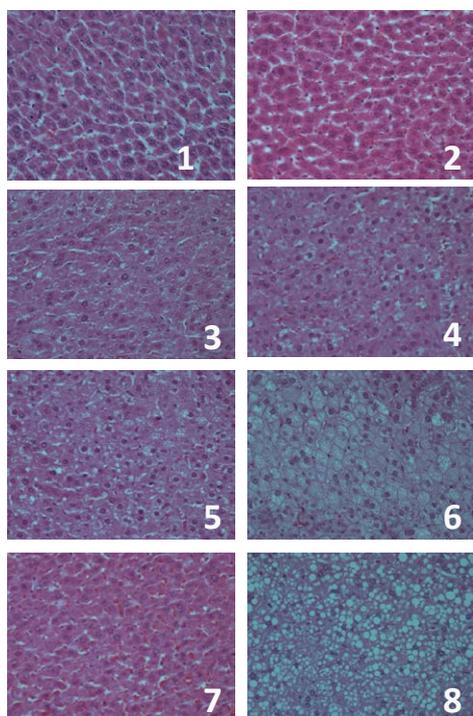


Figure 1: Micrography of livers with different administration times of a hypercholesterolemic diet + 60% fructose N, standard diet and purified water fed intragastrically (1ml/kg) for seven weeks (1); 4D, hypercholesterolemic and 60% fructose for four days (2); 1W, hypercholesterolemic diet and 60% fructose for one week (3); 2W, hypercholesterolemic diet and 60% fructose for two weeks (4); 3W, hypercholesterolemic diet and 60% fructose for three weeks (5); 4W, hypercholesterolemic diet and 60% fructose for four weeks (6); 4WF, 60% fructose for four weeks (7); and 7W, hypercholesterolemic diet and 60% fructose for seven weeks (8).

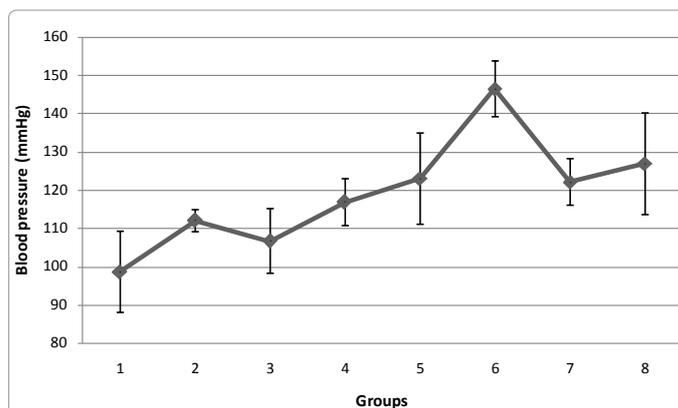


Figure 2: Arterial pressure in rats with metabolic syndrome at different time periods. The groups are as follows: 1) N, 2) 4D, 3) 1W, 4) 2W, 5) 3W, 6) 4W, 7) 4WF, and 8) 7W. Tail SBP were measured with the use of tail-cuff plethysmography in unanesthetized rats (LE 5001-Pressure Meter, Panlab SA, Barcelona, Spain); ANOVA and Student-Newman-Keuls test.

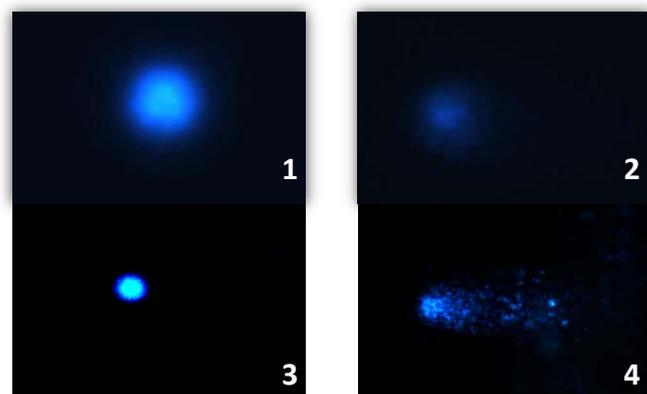


Figure 3: Comet assay micrographs of nucleotides in blood and liver cells from rats with MS. The micrographs are labeled as follows: 1) N blood, 3) N liver, and 4) 7W liver.

SAMPLE	COMET	NUMBER OF COMETS IN 100 NUCLEOTIDES	INDEX
BLOOD	T-	1.50 ± 1.38	1.01
	T+	5.88 ± 2.59*	1.05
LIVER	T-	11.6 ± 4.41	1.02
	T+	75.5 ± 7.12*	2.91

*Significant differences with respect to the control group (N) according to a one-way ANOVA and Student-Newman-Keuls test. The following abbreviations are used: N, standard diet and purified water fed intragastrically (1ml/kg) for seven weeks; 4D, hypercholesterolemic and 60% fructose for four days; 1W, hypercholesterolemic diet and 60% fructose for one week; 2W, hypercholesterolemic diet and 60% fructose for two weeks; 3W, hypercholesterolemic diet and 60% fructose for three weeks; 4W, hypercholesterolemic diet and 60% fructose for four weeks; 4WF, 60% fructose for four weeks; and 7W, hypercholesterolemic diet and 60% fructose for seven weeks

Table 3: Comet assay in blood and liver cells of rats with metabolic syndrome.

fibrogenesis. Other factors, including PPAR mutations and genetic factors, also play a role in hepatic damage [35].

Conclusions

The animal model presented in this study showed evidence for MS in animals fed a hypercholesterolemic diet and 60% fructose for four weeks with the following well defined markers: increased total

cholesterol, LDL cholesterol, atherogenic index, weight gain, and liver weight; decreased HDL cholesterol; hypertension; and defined steatosis, producing an increase in plasma ALP. These well-defined markers were not present in rats fed only 60% fructose for four weeks. In addition, at seven weeks, hyperglycemias as well as increased fat, non-alcoholic fatty liver, genotoxic damage in the liver, and genotoxic damage in the blood were observed. This model closely resembles the pathophysiology of humans who develop MS.

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