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Age-dependent hepatic alterations induced by a high-fat high-fructose diet

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Abstract

Objective The present study aimed to evaluate and clarify how the age at which the intake of a high-fat and high-fructose diet begins can affect animals' livers.

Methods Thirty-eight male wistar rats aged 6 and 12 weeks were fed a high-fat and high-fructose diet for 13 weeks. Inflammatory cytokines, hepatic glycogen, serum and hepatic triacylglycerol and pAkt protein content in the liver were assessed. Percentage of weight gained, and visceral adiposity were also evaluated.

Results Young animal presented increased hepatic triacylglycerol and decreased glycogen, while adult animals had no significant alterations regarding its contents. IL6 and IL10 to IL6 ratio were also altered in young animals exposed to HFHF, while adult animals fed with HFHF had only increases in TNF-α. Both groups which received HFHF had increased serum triacylglycerol and visceral adiposity. However, only young animals gained more relative weight and had greater final body weight, gains which were related to alterations found in hepatic triacylglycerol and glycogen.

Conclusion Age of which consumption begins interferes in how the liver deals with an excess of nutrient and subsequent proinflammatory stimulation, leading to different phenotypes.

Keywords Age-dependent · Inflammation · IL6 · TNF-α · Hepatic triacylglycerol · Hepatic glycogen

Introduction

Obesity physiopathology is largely described and discussed in the literature, and it is even considered an epidemic [1–3]. Developed and developing countries present a high index of an excess of weight in children, adolescents, and adults, despite gender. The values oscillate between 12 and 23% for children and teenagers, and 36 and 38% for adults. These values are growing constantly and gradually since 1980, with no perspective of reduction [4].

The change in the food pattern, regardless of age and social income led to changes in the nutritional status of the global population [5]. The increase in excess of weight is proportional to the increase in the intake of processed and highly processed foods [4–7]. The prevalence of chronic diseases associated with obesity, such as diabetes mellitus, dyslipidemia, hypertension, and metabolic syndrome (MS), as well as the prevalence of chronic hepatic diseases had also a gradual and continuous increase throughout the last decades [8].

It has been shown that the prevalence of non-alcoholic fatty liver disease (NAFLD) to be between 9 and 37% all over the globe. Furthermore, patients with obesity and diabetes tend to present more advanced NAFLD [9], besides, there is a link between the presence of MS and the severity of NAFLD, this association hinders the regression of steatosis [10].

The chronic intake of fat and sugar, generally present in the same meal, for example in the western dietary pattern,

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or the western diet (WD) [11], cause a more constant and continuous weight gain [12, 13] and is highly deleterious to metabolic homeostasis. The WD is commonly used in experimental protocols to induce several chronic metabolic conditions, due to its content of simple carbohydrates and saturated fatty acids [14–17]. Besides the increase in total weight, the composition of the diet also influences and is capable to alter body composition, increasing fat accumulation in most organs or peri-organs deposits generally analyzed [14, 16, 18, 19].

Inflammation causes most of the damage induced by WD, and it is known that saturated fatty acids promote inflammatory response through toll-like receptor 4 (TLR4) pathway activation by binding to CD14-TLR4-MD2 complex [20–23]. Additionally, by promoting the increase of gram-negative bacteria in the intestinal lumen which increases lipopolysaccharides concentration, TLR4 canonical agonist, favoring endotoxemia [24]. In response to the activation of the TLR4 pathway, the cytokines mainly produced are Tumor Necrosis Factor-α (TNF-α), interleukin 6 (IL6) and interleukin 1 beta (IL1β) as pro-inflammatory signals, and Interleukin 10 (IL10) as an anti-inflammatory response [23, 25].

TNF-α promotes inflammatory responses and it also induces another pro-inflammatory mediators' expression. At the same time, IL6 can be seen as an ambiguous cytokine, once it can promote anti-inflammatory responses—IL10 expression—but is in general classified as pro-inflammatory for activating the JAK/STAT3 pathway [25]. In the liver, IL6 can determine or protect from hepatic triacylglycerol accumulation [25], but also, its inhibition improves insulin resistance and consequently glucose and glycogen homeostasis in the same *situ* [26].

Energy is mainly stored in the liver as triacylglycerol and glycogen, and its contents are controlled by metabolic signalization [27]. Triacylglycerol storage may be triggered by glycogen excess which increases expression of genes that promotes lipogenesis, and by substrate excess in energetic metabolism pathway, through activation of carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein type 1c (SREBP-1c) [27–29].

Generally, diet-induced obesity models use a high concentration of saturated fat or sugar, ranging from 30 to 70% of fat and from 25 to 70% of sugar in the diet or diluted on water, or a combination of both [30, 31]. In these studies sucrose or palatable foods are commonly used as a simple carbohydrates source [32], nevertheless, studies focused on hepatic alterations prefer to use high-fructose diets [19, 33]. Fructose per se can induce non-alcoholic hepatic steatosis by mimicking all alterations caused by alcohol intake that leads to alcoholic liver steatosis [34].

Growth and development are accompanied and induced by a natural increase in several pathways which determine mass gain and growth, such as hormone actions and an increase in energy intake [35]. The age or stage of life in which consumption begins is a determinant to the effects observed regarding metabolic alterations [19, 36]. Nevertheless, few studies report differences in hepatic metabolism caused by WD in different ages [36], still, no association was made to the increased rate of development in younger animals in literature.

Thereby the aim of the study was to evaluate the hepatic alterations induced by an high-fat and high-fructose diet (HFHF) beginning in different ages. Thus there was used rats of 6 and 12 weeks were exposed to HFHF or to control diet for 13 weeks.

Materials and methods

Ethics

The present study was approved by the ethics committee of the Universidade Federal de São Paulo under the number of process 2,238,070,515. The experiments were carried out on the Laboratory of Experimental Physical Training (LATREFEX) and on the Laboratory of Metabolic Endocrine Physiology (LaFEM) of the Biosciences Department of the Universidade Federal de São Paulo, Campus Baixada Santista. All procedures were carried out under the ethic principals to animal research, according to the 'Conselho Nacional de Controle de Experimentação Animal' (CONCEA), respecting the federal law N° 1,794/2008.

Experimental protocol

It was used male wistar rats (*Rattus norvegicus*) aged 6 and 12 weeks. The animals received the standard chow diet or the modified diet and water ad libitum. The room temperature was 22 ± 2 °C and it was adopted 12:12 h light/dark cycle. After adaptation, animals were randomly assigned into four groups, young animals were assigned to control diet (CY $n = 8$) and modified diet (HY $n = 11$), the same for adult animals, control diet (CA $n = 10$), and modified diet (HA $n = 9$). The protocol lasted 13 weeks, animals were weighed weekly and diet was replaced each 48 h and weighed before and after being offered. At the end of the protocol, animals were anesthetized locally with lidocaine (10 mg/kg, ip.) and, after 10 min, with thiopental (30 mg/kg, ip.), and beheaded. Trunk blood was collected, liver, mesenteric, retroperitoneal and epididymal fat deposits were removed, weighed and stored at − 80 °C.

Diet

To induce hepatic alterations, a diet rich in saturated fatty acids and fructose (HFHF) was used. It was based on the standard diet and added with 25% lard, 25% fructose, casein and soybean oil and butylated hydroxytoluene (BHT). Until 60 days of age, HY animals received a HFHF diet adapted to growth, then received HFHF adapted to maintenance periods as recommended by AIN-93 guidelines [37], same as HA animals. Specifications of diets are displayed below in Table 1.

Hepatic glycogen

To quantify hepatic glycogen (HGlyc), it was used the micro-method adapted from Balmain, Biggers and Claringbold, 1956 [38]. Samples were digested in 400uL of KOH 30% at 100 °C for 30′, added absolute alcohol 2:1, vortexed, boiled, cooled at 4 °C, and centrifuged at 4500 rpm for 30′. The precipitate was resuspended with 1 mL of trichloroacetic acid (TCA) 5%, vortexed and centrifuged at 4500 rpm for 20′, then the supernatant was added of 1 mL of TCA 5%, vortexed and centrifuged once more at 4500 rpm for 20′. The diluted supernatant was added of Antrone 1:2 (0.2% solution in 95% sulfuric acid) and placed on dry-bath at 100 °C for another 10′. Each sample was read on quartz cuvette at 620 nm. The values found were then compared to a standard curve of dilution.

Table 1 Composition of the diets used in the experimental protocol

	Control diet	HFHF-G	HFHF-M
Components (g/100 g)			
Control diet (Nuvilab CR1®)	100	27.28	33.98
Lard	–	25.00	25.00
Fructose	–	25.00	25.00
Casein	–	20.00	14.00
Soybean oil	–	2.00	2.00
BHT	–	0.02	0.02
Nutrients (g/100 g)			
Carbohydrates	52.50	39.69	42.85
Protein	22.00	26.15	21.48
Lipids	4.00	28.11	28.36
Energy (%)			
Carbohydrates	62.87	30.74	33.44
Fructose	–	19.36	19.51
Protein	26.35	20.26	16.76
Lipids	10.78	49.00	49.80
Saturated fat	–	17.08	17.21
Energy (Kcal/100 g)	334.00	516.45	512.56

Components of each diet, nutritional information, energy contribution of each macronutrient and energy density. (control diet) consumed by all animals during the adaptation period and by control-young and control-adult throughout the experimental protocol. (HFHF-G) consumed by HFHF-Young animals from the begging of the experiment to the 60 post-natal day. (HFHF-M) consumed by the HFHF-Young groups from the 61 post-natal day to the end of the experimental protocol and by the HFHF-Adult groups throughout the experimental protocol

BHT butylated hydroxytoluene

Hepatic triacylglycerol

The content of hepatic triacylglycerol (HTag) was determined through total lipids extraction as described by Folch et al. 1957 [39]. To each sample was added 3,5 ml of a mixture of chloroform, methanol, and water (2:1:0.5), homogenized and centrifuged at 2000 rpm for 10′. The precipitate diluted in chloroform was evaporated and resuspended with 1 mL of Triton 3%. The samples were then analyzed by the colorimetric method using a commercial kit (Labtest®).

Western blotting

Western blotting analysis was performed for hepatic protein extract as previously described by Santamarina et al. 2018 [40] for Akt (1:1200, #4685, Cell Signaling Technology (CST), Danvers, MA, USA), and pAkt (1:1,200, #4060, CST, Danvers, MA, USA). As secondary antibody, it was used Anti-rabbit IgG, HRP-linked (1:20,000, #7074, CST, Danvers, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The liver protein extract was used to assess IL6, IL10, and TNF-α content through immunoenzymatic kits (R&D System, Minneapolis, MN, EUA) following the manufactory instructions.

Statistical analyses

The data was compiled using Microsoft Excel® 2016 and analyzed with SPSS Statistics, Version 22.0. Normal distribution was verified by the Shapiro–Wilk test. Outliers were assessed by Grubb's test. The parametric data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni's as post hoc test, the nonparametric data were analyzed by Kruskal–Wallis test with Dunn's post hoc test. For effect size, it was reported Cohen's f for ANOVA analysis and for post hoc analysis it was reported Hedges' g. Multiple linear and linear regressions were performed to assess predictive factors. The statistically significant limit considered was 5% ($p \leq 0.05$). Data presented with statistically significant differences are marked with '@' if means differed from CY, with '#' if differed from HY as and from CA with '&'.

Results

Murinometric parameters and food intake

Final body weight (FBW) was greater in HY compared to CY ($p = 0.03$; $g = -1.37$), and no difference was found in adult groups (Ad). Young groups (Yn) had a greater percentage of weight gained (PWG) compared to the Ad (CY vs CA $p < 0.0001$; $g = 6.90$, HY vs HA $p < 0.0001$; $g = 5.28$). Moreover, HY had higher PWG than CY ($p = 0.0007$; $g = -1.50$), but no difference was observed comparing CA with HA ($p = 0.31$; $g = -1.29$) (Table 2). Additionally, caloric intake was found to be decreased in both HFHF groups compared to its controls (CY vs HY $p = 0.04$; $g = 1.45$, CA vs HA $p < 0.0001$; $g = 1.91$) and decreased in HA compared to HY ($p < 0.0001$; $g = 1.64$) (Fig. 1a). In the same way, metabolic efficiency was compromised in animals which receive an HFHF diet (CY vs HY $p < 0.0001$; $g = -9.88$, CA vs HA $p < 0.0001$; $g = -6.65$), and more diminished in HY than in HA ($p = 0.003$; $g = 2.73$) (Fig. 1b).

For body composition, it was found a significative increase in the sum of white visceral adipose tissue ($\sum$WAT) (g/100 g), mesenteric fat, retroperitoneal fat, and epididymal fat, in the HH groups, regardless of age (CY vs HY $p < 0.0001$; $g = -2.55$, CA vs HA $p < 0.0001$; $g = -2.22$). No difference was found in relative liver weight (LW) (g/100 g body weight) ($F_{(3,33)} = 2.01$; $p = 0.13$;$f = 0.43$) (Table 2).

White adipose tissue correlated to cytokine expression depending on age. Yn had IL6 positively correlated to FBW ($r = 0.564$; $p = 0.02$) and to $\sum$WAT ($r = 0.715$; $p = 0.001$) which was also correlated positively to HTag ($r = 0.764$; $p = 0.001$) and negatively to HGlyc ($r = -0.565$; $p = 0.01$). Whereas Ad had TNF-α positively correlated to $\sum$WAT ($r = 0.599$; $p = 0.02$).

Table 2 Murinometric parameters and Food intake

Parameters	CY (mean ± SEM)	HY (mean ± SEM)	CA (mean ± SEM)	HA (mean ± SEM)	ANOVA *p* value
Final body weight (g)	384.60 ± 16.74	454.80 ± 15.12[@]	428.70 ± 13.76	461.00 ± 24.14	0.0214*
Percentage of weight gained (%)	126.60 ± 4.82	159.40 ± 7.43[@]	35.67 ± 3.68[@]	50.02 ± 3.21[#]	< 0.0001*
Metabolic efficiency (g weight gained/kcal consumed)	0.0136 ± 0.0012	0.0456 ± 0.0018[@]	0.0074 ± 0.0007	0.0331 ± 0.0024[#,&]	< 0.0001*
Energy consumption (Kcal)	6496 ± 34.16	5952 ± 130.4[@]	6025 ± 65.97	4761 ± 237.6[#,&]	0.0002*
Sum of visceral fat (g/100 g)	4.53 ± 0.34	8.09 ± 0.46[@]	3.44 ± 0.32	7.37 ± 0.74[&]	< 0.0001*
Relative liver weight (g/100 g)	3.23 ± 0.09	3.06 ± 0.09	3.29 ± 0.13	3.00 ± 0.07	0.1317

CY control-Young, *HY* HFHF-Young, *CA* control-adult, *HA* HFHF-adult, *SEM* standard error of mean. [@]differed from, *CY*, [#]differed from HY, [&]differed from CA

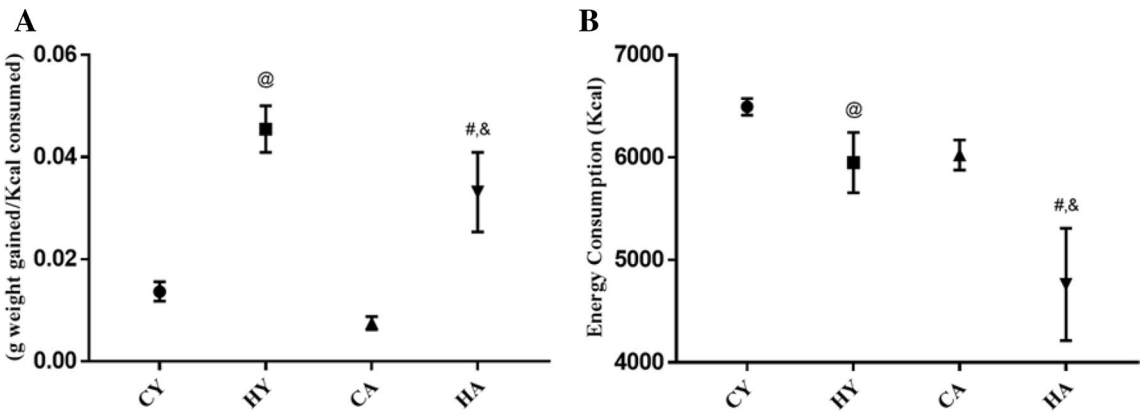

Fig. 1 Graphical representation of mean ± 95% confidence interval. **a** Metabolic efficiency, grams of weight gained per kilocalorie consumed. **b** Energy consumption throughout the protocol. *CY* control-young, *HY* HFHF-young, *CA* control-adult, *HA* HFHF-adult. [@]Differed from CY, [#]differed from HY, [&]differed from CA

Table 3 Hepatic parameters and serum triacylglycerol

Parameters	CY (mean ± SEM)	HY (mean ± SEM)	CA (mean ± SEM)	HA (mean ± SEM)	ANOVA *p* value
Hepatic Glycogen (mg/g)	139.90 ± 22.95	58.13 ± 14.42[@]	134.80 ± 14.41	134.30 ± 17.44[#]	< 0.0001*
pAkt/Akt (A.U.)	100.00 ± 6.45	81.31 ± 8.64	131.80 ± 24.15	138.10 ± 29.89	0.1783
Hepatic Triacylglycerol (mg/g)	22.37 ± 1.82	46.58 ± 6.40[@]	27.81 ± 1.71	37.01 ± 4.46	0.0014*
IL6 (pg/mg)	71.75 ± 3.77	122.30 ± 14.65[@]	107.00 ± 9.51	130.70 ± 11.68	0.0042*
IL10 (pg/mg)	117.60 ± 9.32	132.80 ± 9.77	119.10 ± 3.74	112.10 ± 5.46	0.2877
TNF-a (pg/mg)	10.28 ± 0.74	12.64 ± 0.92	12.05 ± 1.34	19.02 ± 2.57[#,&]	0.0022*
IL10/IL6	1.63 ± 0.09	1.12 ± 0.18[@]	1.18 ± 0.10[@]	0.91 ± 0.09	0.0009*
Serum Triacylglycerol (mg/dl)	129.8 ± 10.68	169.20 ± 9.29[@]	88.29 ± 5.03[@]	137.90 ± 8.020[&]	< 0.0001*

CY control-young, *HY* HFHF-young, *CA* control-adult, *HA* HFHF-adult, *SEM* standard error of mean. [@]differed from CY, [#]differed from HY, [&]differed from CA

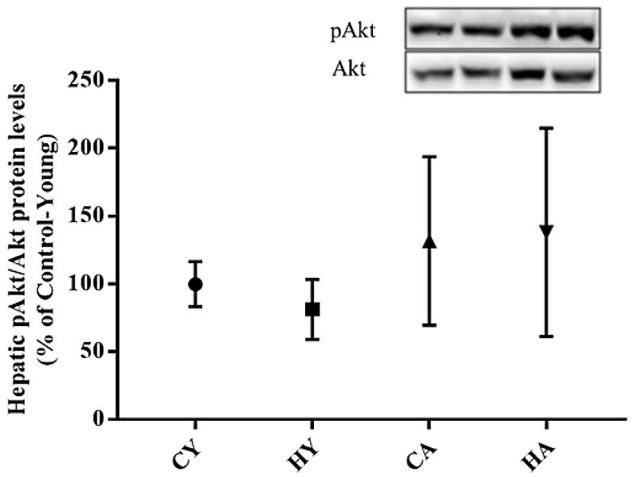

Fig. 2 Graphical representation of Mean ± 95% Confidence Interval of hepatic phosphorylated Akt relative to total Akt protein content detected by Western Blotting. Results are expressed as percentages of control-young group in arbitrary units. *CY* control-young, *HY* HFHF-young, *CA* control-adult, *HA* HFHF-Adult

Table 4 Multiple linear regression

Model		Coefficients		Significance	VIF
		β	*t*		
1	(Constant)		5.9479	< 0.0001	
	Diet	− 0.3564	− 2.2568	0.0304*	1.0000
	(Constant)		2.871	0.0070	
	Diet	− 0.3313	− 2.1807	0.0362*	1.0067
	Age	0.3066	2.0183	0.0515	1.0067
2	(Constant)		5.2114	0.0001	
	Diet	− 0.6174	− 3.139	0.0063*	1.0000
3	(Constant)		3.8705	0.0012	
	Diet	− 0.0047	-0.0196	0.9846	1.0000

Linear regression. HGlyc and diet

Model 1. Multiple Linear Regression. HGlyc and Diet ($C = 1$; HFHF = 2). Age ($Y = 1$; $A = 2$). All groups

Model 2. Linear Regression. HGlyc and Diet (C = 1; HFHF = 2). Yn group

Model 3. Linear Regression. HGlyc and Diet (C = 1; HFHF = 2). Ad group

Hepatic inflammatory *Profile*

It was observed an increase in pro-inflammatory cytokines in HFHF groups (HH). HY had an increased expression of IL6 ($p = 0.01$; $g = -2.87$) and lower expression of IL10 relative to IL6 when compared to CY ($p = 0.02$; $g = 1.35$). However, in the HA group, TNF-α was increased when compared to its control for diet, CA ($p = 0.01$; $g = -1.22$) and to HY ($p = 0.02$; $g = -1.20$) (Table 3).

There was a non-significant increase in IL6 comparing CA with CY (107.00 vs 71.75; $p = 0.12$; $g = -2.29$) and a significative decrease in expression of IL10 relative to IL6 ($p = 0.03$; $g = 1.49$) (Table 3).

Hepatic glycogen

It was found lower hepatic glycogen content (HGlyc) In the HY group when compared to CY ($p = 0.01$; $g = 1.42$) and HA ($p = 0.01$; $g = -1.49$). There was no significative difference in Akt phosphorylation ratio, pAkt/Akt (pARatio) (CY vs CA $p > 0.99$; $g = -0.68$, HY vs HA $p = 0.23$; $g = -0.97$) (Table 3; Fig. 2). However, there was a positive correlation between pARatio and HGlyc in HY ($r = 0.82$; $p = 0.01$). Even, serum glucose was unchanged among groups ($F_{(3,31)} = 2.11$, $p = 0.12$, $f = 0.45$).

Still, HFHF was a negative predictor for HGlyc independently of age (C = 1; HFHF = 2), though, age might have influenced ($p = 0.051$), then, diet was a negative predictor for HGlyc in Yn ($p = 0.01$), but not for Ad ($p = 0.98$) (Table 4). Additionally, HGlyc was negatively

correlated with PWG in Yn ($r = 0.50$; $p = 0.04$), but not in Ad ($r = 0.06$; $p = 0.81$).

Hepatic Glycogen and inflammation

The IL10/IL6 ratio was not found to be a preditor for HGlyc in all groups, but as age could be interfering on the analysis ($p = 0.06$) and as IL10/IL6 ratio and HGlyc have presented a significative difference in Yn animals (Table 3), it was performed a separate analysis for Yn and Ad groups. The IL10/IL6 ratio was found to be a positive predictor for HGlyc in Yn ($p = 0.05$), but not in Ad ($p = 0.41$) (Table 5). TNF-α was found to be a preditor for HGlyc ($p = 0.03$), but, as diet interfered on the analysis ($p = 0.01$), groups were separated per diet. TNF-α was a predictor in HH groups ($p = 0.001$) independently from age (Table 6).

3.4 Serum and hepatic triacylglycerol

Hepatic triacylglycerol

Fat infiltration It was found greater HTag content in the HY group when compared to CY ($p = 0.001$; $g = -1.72$) and was observed a small nonsignificant increase in the HA group when compared to the CA ($p = 0.46$; $g = -0.91$) (Table 3). HTag was positively correlated with PWG in Yn ($r = 0.72$; $p = 0.002$), but not in Ad ($r = 0.31$; $p = 0.24$).

No animals in Ct groups had significant hepatic fat infiltration (≥ 5%). Half of HY (4; $n = 8$) and a quarter of HA (2; $n = 8$) presented 5% or more fat infiltration in its livers. Similarly, to HTag content, HY significantly differed from CY ($p = 0.04$ $g = -1.25$), and despite its control group had no animals with more than 5% content, there was no significative difference between Ad groups ($p = 0.83$; $g = -0.72$) (Table 7).

Hepatic Triacylglycerol and inflammation IL10/IL6 was not a predictor for HTag in all groups, but, as the diet exert an influence on the analysis ($p = 0.001$), the groups were once more separated. IL10/IL6 was a positive predictor for

Table 5 Multiple linear regression

Model		Coefficients		Significance	VIF
		β	*t*		
1	(Constant)		2.3236	0.0274	
	IL10/IL6	0.2993	1.6892	0.1019	1.0000
	(Constant)		0.0774	0.9389	
	IL10/IL6	0.3910	1.8078	0.0818	1.6146
	Age	0.3622	1.9343	0.0636	1.2102
	Diet	−0.0914	−0.4564	0.6517	1.3842
2	(Constant)		−0.7956	0.4417	
	IL10/IL6	0.7149	3.5421	0.0041*	1.0000
	(Constant)		0.5676	0.5817	
	IL10/IL6	0.5549	2.2019	0.0499*	1.5729
	Diet	−0.2652	−1.0523	0.3153	1.5729
3	(Constant)		4.1938	0.0008	
	IL10/IL6	−0.2441	−0.9751	0.345	1.0000
	(Constant)		2.3875	0.0316	
	IL10/IL6	−0.2486	−0.851	0.4091	1.2705
	Diet	−0.0096	−0.0329	0.9742	1.2705

IL6/IL10 and diet

Model 1. Multiple Linear Regression. HGlyc and IL10/IL6. Age (Y = 1; A = 2). Diet (C = 1; HFHF = 2). All groups

Model 2. Multiple Linear Regression. HGlyc and IL10/IL6. Diet (C = 1; HFHF = 2). Yn group

Model 3. Multiple Linear Regression. HGlyc and IL10/IL6. Diet (C = 1; HFHF = 2). Ad group

Table 6 Multiple Linear Regression

Model		Coefficients		Significance	VIF
		β	*t*		
1	(Constant)		2.71	0.0114	
	TNF-α	0.2400	1.3083	0.2014	1.0000
	(Constant)		3.1017	0.0046	
	TNF-α	0.4901	2.3507	0.0266*	1.6023
	Age	0.0494	0.272	0.7877	1.2144
	Diet	−0.545	−2.8088	0.0093*	1.3879
2	(Constant)		3.6216	0.0028	
	TNF-α	−0.2444	−0.9429	0.3617	1.0000
	(Constant)		3.2367	0.0065	
	TNF-α	−0.2216	-0.7896	0.4439	1.0950
	Age	−0.0774	−0.2758	0.787	1.0950
3	(Constant)		−1.7601	0.1038	
	TNF-α	0.8548	5.7049	0.0001*	1.0000
	(Constant)		−2.1837	0.0515	
	TNF-α	0.7405	4.2946	0.0013*	1.3875
	Age	0.2161	1.2533	0.2361	1.3875

Hepatic Glycogen and TNF-α

Model 1. Multiple Linear Regression. Hepatic Glycogen and TNF-α. All groups

Model 2. Multiple Linear Regression. Hepatic Glycogen and TNF-α. Ct groups

Model 3. Multiple Linear Regression. Hepatic Glycogen and TNF-α. HH groups

Table 7 Fatty liver

Fatty liver	CY	HY	CA	HA
Absence (< 5%)	8	4	8	6
Presence (> 5%)	0	4	0	2

All groups

Table 8 Multiple linear regression

Model		Coefficients		Significance	VIF
		β	*t*		
1	(Constant)		3.6815	0.0011	
	IL10/IL6	− 0.0494	− 0.2473	0.8067	1.0000
	(Constant)		− 0.5396	0.5947	
	IL10/IL6	0.2860	1.3220	0.1992	1.7934
	Age	0.0362	0.1858	0.8542	1.4571
	Diet	0.7088	3.8728	0.0008*	1.2833
2	(Constant)		6.3379	< 0.0001	
	IL10/IL6	− 0.4539	− 1.8369	0.0892	1.0000
	(Constant)		2.0704	0.0606	
	IL10/IL6	− 0.2094	− 0.6446	0.5313	1.7685
	Age	0.3709	1.1417	0.2758	1.7685
3	(Constant)		0.3549	0.7300	
	IL10/IL6	0.7289	3.3669	0.0072*	1.0000
	(Constant)		0.7921	0.4487	
	IL10/IL6	0.6587	2.7206	0.0236*	1.1912
	Age	− 0.1752	− 0.7235	0.4878	1.1912

Model 1. Multiple Linear Regression. HTag and IL10/IL6. Age (Y = 1; A = 2). Diet (C = 1; HFHF = 2). All groups

Model 2. Multiple Linear Regression. HTag and IL10/IL6. Age (Y = 1; A = 2). Ct group

Model 3. Multiple Linear Regression. HTag and IL10/IL6. Age (Y = 1; A = 2). HH group

HTag only in the HH groups independently of age ($p = 0.02$) (Table 8).

Serum triacylglycerol

Serum Triacylglycerol (STag) was increased in HH groups compared to its controls (CY vs HY $p = 0.01$ $g = -1.23$; CA vs HA $p = 0.004$ $g = -2.47$), and CA was lower than CY ($p = 0.02$; $g = 1.63$). HY had a non-significantly increased regarding HA (169.20 vs 137.90; $p = 0.06$; $g = 1.08$) (Table 3). STag positively correlated to PWG and ∑WAT only in HY ($r = 0.63$ $p = 0.04$; $r = 0.79$ $p = 0.004$).

Discussion

The literature describes western diets as responsible for weight gain [15, 19]. Although the present study found increased weight in animals which started HFHF intake when young, HFHF-Young (HY) differed from control-young (CY) group in body weight and weight gained, but control-adult (CA) was similarly to HFHF-adult (HA). Collectively, Lozano et al., 2016 [19] reports a significative weight gain in wistar rats on high-fat (HFat) and HFHF, but not on high-fructose (HFru). Woodie and Blythe 2017 [15] saw the same increase for Sprague–Dawley rats in HFat.

Younger animals had greater weight gain than adults. de Castro et al. 2013 [36] found the same difference even in control animals, young animals which consumed HFru had lower body weight, but similar to controls if consumed HFat [36]. In contrast, adult animals had lower weight gain when consumed HFat, but no alteration if consumed HFru. Balakumar et al. 2016 [16] findings corroborate with ours, once after 4 months, adult wistar rats had no alteration in FBW on HFat, HFru nor HFHF.

Fat is more responsible for weight gain in rats consuming western diets [19]. This obesogenic effect can be exacerbated by the addition of fructose [16]. Literature reports increased fat deposits in young rats consuming a diet high in fat [15, 16, 36], fructose [15, 36], and both [16], and, increased total body lipids [41] and adiposity [16] in adult rats consuming HFat or HFHF. However, similar ∑WAT in adult rats on HFat or HF [36].

Serum triacylglycerol increase in HY is also reported in young animals exposed to HFHF for 10 and 15 weeks [22, 29]. While 4 weeks old Fischer rats had no increase after 13 weeks of HFru [36], 6 weeks old Sprague–Dawley rats presented an increase after 10 weeks [15]. Even, both above-cited studies observed no alterations after HFat consumption. Balakumar et al. saw a greater STag in adult animals after 4 months of HFHF, a result found acutely by Crescenzo et al. after HFHF [41].

The increase in pro-inflammatory cytokines also points to an age-dependent inflammatory response. Whereas HY had an increase in IL6 compared to CY, HA had an increase in TNF-α. Additionally, IL10/IL6 was decreased in HY and CA compared to CY, with no alterations in IL6 content for CA, suggesting a natural deficit in IL10 compensation with time.

Hepatic IL6 and TNF-α have not changed in 30 days old mice on HFat for 16 weeks [42], but these cytokines were increased in serum of 6 weeks old mice exposed to HFHF for 4 weeks [43]. Chronically, serum TNF-α was increased in 8 weeks old rats exposed to HFat and HFHF, but not to HFru [19]. Still, Aragno et al. [29] reported serum and hepatic TNF-α increase in young wistar rats after 15 weeks of a HFat diet added with 10% fructose. This result differed from what was observed in the present study, what may be due to water-based administration.

Increase in IL6 is implicated in insulin resistance and obesity, while its inhibition improved hepatic steatosis in HFat fed mice [44]. Hassan et al. [25] discuss the paradox findings of IL6 and hepatic lipids. While some studies report its capacity to protect the liver from fat accumulation, there is also a body of evidence that supports its effect on deregulating lipids metabolism in the same situ. After all, IL6 could

protect from hepatic triacylglycerol accumulation, but its overexpression might aggravate it [25].

IL6 and TNF-α were positively correlated to ∑WAT, but one in each age, IL6 in young and TNF-α in the adult animals. HY had an increase in IL6 expression and a decrease in IL10 relative expression, what could be associated with the intrahepatic lipid accumulation. Moreover, IL10/IL6 was positively correlated with HTag considering all groups, also, it was a positive predictor for animals consuming HFHF. As no difference was observed in IL10 expression among groups, the inflammatory stimulus is more likely to be the common factor.

Glycogen and triacylglycerol are the main liver energy storage. The results found for hepatic triacylglycerol in the HY group could be triggered by a glycogen accumulation [27]. Nonetheless, as HGlyc was decreased, tissue-specific insulin resistance should be considered, stimulated by IL6, an excessive load of fructose and/or an excess of fat entering hepatocytes [16, 18, 26–28, 34].

HFHF and HFat diets can increase Tag in the liver of young rats [19], but glycogen decrease is a characteristic attributed to more severe cases of fatty liver diseases, as cirrhosis [45]. A possible confounder could be glycogen depletion by fasting, but animals were not fastened before euthanasia. Crescenzo et al, 2014 [41] observed in 12 weeks old Sprague–Dawley rats consuming HFat for 2 weeks lowered HGlyc and increased HTag. Though, when receiving HFHF, animals presented intermediary HTag and HGlyc contents, between control and HFat. Thus, fructose addition prevented the decrease of HGlyc acutely in adult animals [41].

Different inflammatory stimuli were associated with HGlyc response in HY group. Firstly, HY had insufficient anti-inflammatory compensation—decreased IL10/IL6 ratio—which was a positive predictor for hepatic glycogen at the younger age. Thus, uncompensated inflammation regarding IL6 signaling might have also affected HGlyc. Secondly, it had lower TNF-α, a positive predictor for HGlyc in HFHF consuming animals. Interestingly, results found in HY—HGlyc depletion and HTag accumulation—are shown as a consequence of acute HFat exposure in adult animals [41].

Conversely, fructose and SFA action on hepatocytes is a hypothesis to be considered. Saturated fatty acids and fructose-enriched diets modify and impair cerebral and systemic energetic homeostasis, besides, they increase hepatic fatty acid synthesis [15, 32, 41, 46]. Fructose can decrease HGlyc content by gluconeogenesis independently of insulin signaling [34, 36]. Even more, fructose is not converted to glycogen [23], despite being almost entirely taken from circulation by the liver, it becomes a substrate for de novo lipogeneses, also, it may favor hepatic inflammation [34, 36]. HFHF diet fed animals have increased carbohydrate-responsive element-binding protein (ChREBP) [34], favoring glycogen breakdown. Concomitantly, there is an increase in de novo lipogenesis due to higher subtract availability, and in an increase in the activation of sterol regulatory element-binding protein 1c (SREBP-1c) [16, 18, 34].

TNF-α induces IL6 production [26]. We found an increase in IL6 in young animals, not fully compensated by IL10 expression, and, an increased TNF-α in adult animals which would induce IL6 expression, but HA had a proportional IL10 compensation. IL6 increase is a signal of the acute-phase response of hepatic injury [25], indicating a constant increase in primary response in HY.

Perhaps, adult animals have a greater capacity to adapt to aggression over time, increasing inflammatory and anti-inflammatory preferable pathways, not overexpressing IL6 under TNF-α stimuli, but still responding to the aggression. The TNF-α increase in HA could indicate a more consistent inflammatory response to diet. Overall, animals starting younger consuming HFHF were more propense to alterations in energy storage metabolism, but both propense to inflammation, despite differing in response.

Diet components can induce metabolic alteration depending on age, whereas young rats are more affected by a HFat, adults are more affected by an HFru [36]. But as the present study combines these components to comprehend more the WD factors, the isolation of effects cannot be done. Nonetheless, literature data support that saturated fatty acids and simple carbohydrates can worsen [41] and complement [19] the effects of one another, and even compete for action, presenting an intermediate phenotype of the induced alterations [19, 41].

Finally, in our knowledge, the pattern observed in HY which was an increased HTag and decreased HGlyc—both correlated with the percentage of weight gained—was not reported before. Inflammation was present in both ages but differed in overexpression of marker which was related to adiposity, each cytokine related to ∑WAT in the age it was altered. There is a possibility that the increase in growth and development signaling, was not followed by a proportional increase in adaptive mechanisms, as homeostasis maintenance depends on the ability to adapt to new situations and protect from aggression. We hypothesize that these results are consequences of the higher propensity to alterations, attributed to greater lability in young rats' metabolisms. This lability is intrinsic to its stage of development which presents increased growth and development signaling. Therefore, this 'window of opportunity' could be used to cause an enhanced increase in hepatic alterations induced by diet, concurring with literature, alterations presented by animals exposed to WD depends on age.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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