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Early onset and progression of non-alcoholic fatty liver disease in young monosodium L-glutamate-induced obese mice

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Original Article

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Abstract

Monosodium L-glutamate (MSG)-induced obesity is a useful model for non-alcoholic fatty liver disease (NAFLD) studies. However, there is limited data on its initiation and progression. Thus, this study aimed to characterize the onset of metabolic and histopathological features of NAFLD and its progression to non-alcoholic steatohepatitis (NASH) in this model. To perform this study, Swiss mice pups were neonatally injected with MSG (4 g/kg/day, s.c.) or equiosmolar saline and followed up to 60, 120 or 180 days old. At each age, blood, liver, as well as periepididymal and retroperitoneal fat pads were collected for morphometric, biochemical and histological analyses, the later according to NAFLD activity score. MSG mice presented hypertriglyceridemia and central obesity at all ages, but peripheral insulin-resistance was verified only in 120- and 180-day-old mice. Hepatic total fat and triglycerides content were higher in MSG mice at all ages. Accordingly, histopathological analysis showed that 60-day-old MSG mice had microvesicular steatosis with occasional ballooning, which evolved into NASH from 120 days old. Retroperitoneal fat accumulation was the only variable to independently correlate with NAFLD activity total score upon multivariate analysis ($R^2 = 71.45\%$). There were no differences in IL-6 and TNF-α serum levels among groups. Overall, this study shows that NAFLD is a precocious outcome in MSG-obese mice, whereas the period comprised between 60 and 120 days old seems to be a crucial metabolic window for comprehending pathophysiological events involved in NAFLD-to-NASH progression in this model.

Introduction

Non-alcoholic fatty liver disease (NAFLD), defined as the cytoplasmic lipid accumulation inside hepatocytes in the absence of relevant alcohol intake,[1] has been considered the main hepatic manifestation of metabolic syndrome (MetS) and whose prevalence has reached epidemic levels worldwide.[2] Upon sustained injury, hepatocytes start releasing pro-inflammatory cytokines that lead to macrophage infiltration and disease progression to non-alcoholic steatohepatitis (NASH).[3] These histopathologic and biomolecular changes have been tentatively explained by the 'Two Hits Hypothesis' for NAFLD/NASH, according to which the first hit corresponds to steatosis and the second one to the joint factors oxidative stress and cytokines release.[3]

Histopathological criteria for NASH diagnosis include mandatory presence of steatosis, ballooning degeneration and lobular inflammation, with or without perisinusoidal fibrosis on hepatic lobule zone 3.[4] However, physiopathological mechanisms underlying this process are still barely understood, meanwhile available animal models not fully embrace human NAFLD/NASH features.[5] According to Kanuri and Bergheim,[6] an ideal animal model should meet two basic criteria: histopathological pattern similar to human NAFLD and steatohepatitis onset occurring under a dysfunctional metabolic milieu. More recently, it has been pointed out that most NAFLD/NASH models do not evolve to more severe stages of liver disease.[7]

Neonatal injection of monosodium L-glutamate (MSG) damages hypothalamic nuclei, for example, arcuate nucleus,[8,9] leading to deficient growth hormone (GH) secretion, as well as autonomic unbalance characterized by decreased sympathetic and increased parasympathetic toni.[10–12] Consequently, MSG-obese rodents show hyperinsulinemia and reduced serum levels of insulin-growth factor 1 (IGF-1) at ages as early as 4 weeks old,[13–15] which precede the development of dyslipidemia, glucose intolerance, hyperleptinemia insulin resistance, central obesity and type II diabetes mellitus (T2DM) at adulthood.[16–19] Noteworthy, early infant GH deficiency and hyperinsulinemia are thought to predispose to a range of late-in-life chronic conditions due to metabolic programming.[20] Thus, those gathered features support MSG

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obesity as a reliable model to study MetS-related comorbidities under the developmental origins of health and disease concept.

In a previous study, we showed that 16-week-old MSG rats had NAFLD associated to hypertriglyceridemia.[21] Moreover, it is well documented that 24-week-old MSG mice acquire acute NASH with human histopathologic features,[22] which evolved into cirrhosis around 54 weeks old.[17] Other studies have also described NAFLD-to-NASH development in MSG mice, but generally in adult to middle-aged animals.[22,23] However, given the early metabolic disturbances abovementioned, we hypothesized that NAFLD onset and progression indeed occur at younger ages. Thus, in this study we sought to characterize the onset of NAFLD in young MSG mice (60 days old) and its evolution into NASH at adulthood, exploring metabolic and histopathological features at three distinct ages.

Methods

MSG neonatal treatment and obesity evaluation

Male Swiss mice (*Mus musculus*) pups ($n = 18$) were given 4 g/kg/day of MSG (MSG; Sigma Aldrich, St. Louis, MO, USA) via subcutaneous on alternate days during the first 10 days of life. Control animals (CTR, $n = 19$) were given a similar volume of equiosmolar saline solution. Animals were obtained from the Center Animal Facility at Federal University of Maranhão. They were kept at a temperature of 23 ± 2°C, for a 12-h light/dark period, and given water and food *ad libitum*. Besides, animals were weighed twice a week for assessment of weight gain, and at each 30 days of life, Lee Index was determined, which is calculated by dividing the cube root of the body weight (g) by the nasoanal length (cm).[24] MSG and CTR groups were divided into three subgroups according to euthanizing age: 60, 120 and 180 days old ($CTR/MSG_{60,120,180}$).

At the predetermined ages, after overnight fasting, the animals were anesthetized (10 mg/kg xylazine, 40 mg/kg ketamine), weighed for morphometric assessment and subjected to laparotomy to collect periepididymal and retroperitoneal fat pads (PFP and RFP, respectively), and liver. Blood was collected by retro-orbital sinus puncture. All of the protocols were in accordance with international guidelines for animal care and welfare, and were approved by the Committee for Animal Care and Welfare (CEUA) of the Federal University of Maranhão, rule no. 001/2009.

Biochemistry and insulin resistance by TyG index

The blood samples were coagulated and centrifuged to separate the serum. The serum total cholesterol, triglycerides (TG), glucose levels were analyzed using spectrophotometry with the assistance of commercial kits from Labtest (Labtest, Minas Gerais, Brazil), following manufacturer's instructions. Insulin resistance was assessed by TyG Index analysis, obtained by the following formulae: Ln[fasting triglycerides (mg/dl) × fasting glucose (mg/dl)/2].[25]

Cytokines serum concentrations by flow cytometry

We assessed cytokines (TNF-α and IL-6) serum concentration by cytometric bead assay technique. All utilized reagents were obtained from Mouse Inflammation Kit (Becton Dickinson Biosciences, San Jose, CA, USA). After reading the samples in a flow cytometer, data were analyzed by FCAP Array 3.0 (Becton Dickinson Biosciences), where values were expressed in pg/ml for each cytokine.

Hepatic lipid profile assessment

Samples from liver were homogenized in a chloroform/metanol (2/1, v/v) solution. The homogenate rested overnight and, on the following day, it was filtered with common filter paper. 0.9% saline solution was added to the final solution (1/5, v/v), and the final content was mixed by inversion. After 2 h on rest, the final content was centrifugated (1000 rpm, 5 min). Aqueous phase was discarded and the other one dried in a Petri plate. Thereafter, the amount of fat in each sample was calculated. Total fat (TF) was resuspended in 1 ml of Triton X-100/metanol (2/1, v/v) for TG and TC measurement as described above.

Liver histopathology analysis

Samples from hepatic tissue were fixed in 10% phosphate-buffered formalin solution and they were analyzed by light microscopy either after hematoxilin–eosin (HE) or Masson's Trichrome staining. NAFLD activity score (NAS) was applied during analysis. This score was validated by Kleiner *et al.*[26] and it is based in a semi-quantitative analysis of the three definer criteria of NASH: steatosis (0–3), ballooning (0–3), and lobular inflammation (0–2). Total score is a value that ranges from 0 to 8, which indicates a prognostic status in man/animal liver. Scores >6 indicate NASH; from 3 to 5, borderline (either it can be or not be NASH); and from 0 to 2, it is not NASH. Moreover, there is a fibrosis score, from 0 to 4, which is not considered for total NAS pointing.[26]

Statistical analysis

CTR and MSG groups were compared using Student's *t*-test, while MSG subgroups were compared by ANOVA with Newman-Keuls' post-test. The significance level was established as $P < 0.05$. The results were expressed as the mean ± standard error of the mean (except for cytokines, expressed as median) and analyzed using the software Prism 5 (GraphPad, San Diego, CA, USA). Multivariate analysis was performed using the software Stata 14 (StataCorp, Lakeway Drive, TX, USA) for assessing the independent variable related to NAS total value among the following:[27] group, age, TG, fasting glycemia, RFP, PFP, Lee Index, hepatic triglycerides concentration, total fat in liver.

Results

Timing of MetS development in MSG group

MSG_{60} mice were lighter than CTR_{60} because of their shorter nasoanal length, but had RFP and PFP storages at least 2-fold higher, depicting their increased adiposity, which was further supported by the elevated value of Lee Index (Table 1). As showed in Fig. 1a and 1b, MSG_{60} mice exhibited increased serum levels of TG (50.86 ± 10.75 mg/dl) and total cholesterol (127.7 ± 5.8 mg/dl) as compared with CTR_{60} (30.45 ± 2.74 mg/dl and 93.33 ± 3.27 mg/dl, respectively), $P < 0.05$. On the other hand, MSG_{60} were hypoglycemic (90.0 ± 5.9 mg/dl) in comparison with CTR_{60} (160.0 ± 8.5 mg/dl), $P < 0.05$ (Fig. 1c). Calculation of TyG Index for both groups did not suggest impairment of insulin sensitivity on MSG_{60} (Fig. 1d), which supports that at this age MSG mice were obese but did not develop MetS.

Assessment of the abovementioned parameters showed that weight gain of MSG mice was accelerated, as MSG_{120} body weight did not differ from CTR_{120}, but became higher on MSG_{180} (Table 1). A similar acceleration was observed in serum glucose levels, which were decreased in MSG_{60}, but raised in both MSG_{120}

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Table 1. Morphometric parameters of control and monosodium L-glutamate (MSG)-obese mice

	60 days		120 days		180 days	
	Control	MSG	Control	MSG	Control	MSG
BW (g)	29.3 ± 0.9	21.7 ± 1.3*	32.9 ± 1.2	31.7 ± 1.7	40.0 ± 2.9	48.4 ± 3.2*
Nasoanal length (cm)	9.6 ± 0.1	7.6 ± 0.1*	10.4 ± 0.1#	8.3 ± 0.1*#	10.6 ± 0.2#	9.3 ± 0.3*#§
Lee Index ($g^{1/3}$/cm)	320.2 ± 2.2	362.9 ± 2.9*	307.5 ± 3.3	365.1 ± 2.2*	321.9 ± 5.1	381.9 ± 3.6*
RFP (g/100 g BW)	0.42 ± 0.06	0.78 ± 0.05*	0.28 ± 0.04	1.33 ± 0.09*#	0.65 ± 0.21	1.43 ±0.10*#
PFP (g/100 g BW)	0.92 ± 0.06	2.80 ± 0.12*	0.85 ± 0.09	4.93 ± 0.22*#	0.78 ± 0.24	3.40 ± 0.15*#§
Liver (g/100 g BW)	4.54 ± 0.12	3.17 ± 0.13*	4.89 ± 0.27	3.33 ± 0.09*	4.53 ± 0.25	2.94 ± 0.15*

RFP, retroperitoneal fat pad; PFP, periepydidimal fat pad; BW, body weight.
$n = 5$–7, mean ± S.E.M., Student's test t for MSG *v.* Control at the same age; ANOVA with Newman-Keuls' post-test for MSG/Control subgroups. *$P < 0.05$ *v.* Control (at the same age), #$P < 0.05$ *v.* 60 days (at the same group), §$P < 0.05$ *v.* 120 days (at the same group).

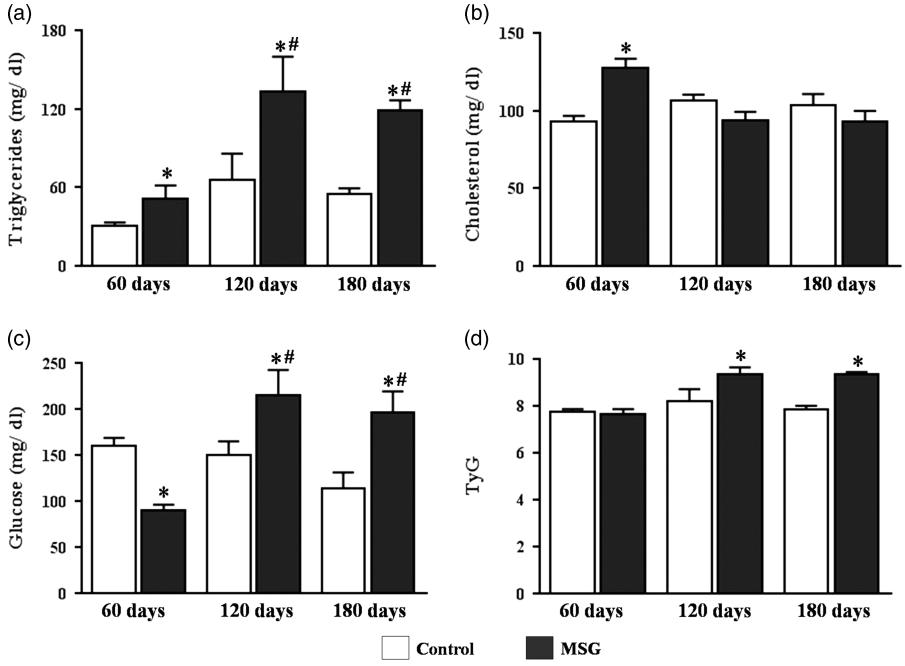

Fig. 1. Serum profile and insulin resistance evaluation through TyG index. Swiss mice received monosodium L-glutamate (MSG) on the first 10 days of life and were euthanized on the 60th, 120th and 180th day of life. Serum triglycerides (*a*), total cholesterol (*b*), fasting glycemia (*c*) were evaluated. TyG Index (*d*) was calculated from fasting triglyceridemia and glycemia values. $n = 5$–7, mean ± S.E.M., *$P < 0.05$ *v.* Control (at the same age), #$P < 0.05$ *v.* 60 days (at the same group). Student's *t*-test for MSG *v.* Control at the same age; ANOVA with Newman-Keuls' post-test for MSG/Control subgroups.

(214.9 ± 27.2 mg/dl) and MSG_{180} (195.8 ± 23.6 mg/dl) groups in comparison with their controls (CTR_{120}: 150.6 ± 14.1 mg/dl; CTR_{180}: 113.9 ± 17.1 mg/dl) (Fig. 1c). Besides glucose, serum TG reached levels as high as twice their controls at both 120 and 180 days old (Fig. 1a), whereas total cholesterol levels did show no difference between groups (Fig. 1b). Accordingly, TyG Index value was significantly increased at both ages (Fig. 1d), which added to the elevated values of Lee Index (Table 1) jointly support the progression of MSG metabolic disturbances toward MetS onset, as they were obese and further became hyperglycemic, hypertriglyceridemic and insulin resistant.

Hepatic lipid accumulation and NAFLD-to-NASH progression in MSG mice

Livers from control mice were heavier than those from MSG at all ages (Table 1), whereas the latter exhibited higher hepatic total fat accumulation at all ages (Fig. 2c). Hepatic TG content was significantly elevated throughout the evaluated ages, reaching values four-fold higher than controls at 180 days old (CTR_{180}: 31.1 ± 5.5 mg/g of liver and MSG_{180}: 126.6 ± 15.7 mg/g of liver), $P < 0.05$ (Fig. 2a). Surprisingly, hepatic total cholesterol levels did not differ between groups, but rather showed a parallel age-dependent increase in both groups (Fig. 2b).

Assessment of NAFLD activity score demonstrated that MSG_{60} already developed NAFLD, mainly characterized by microvesicular steatosis and occasional cellular ballooning (Fig. 3). In MSG_{120} group, livers exhibited inflammatory foci of polymorphonuclear neutrophils and macrophages infiltration, besides mild macrovesicular steatosis with ballooning increase, causing liver disease to evolve into NASH (Fig. 3). This condition was aggravated in MSG_{180}, with a two-fold greater steatosis score (Fig. 3) and other histopathological findings, such as micro-granulomes and glycogenated nuclei (Fig. 4a and 4b). No fibrosis

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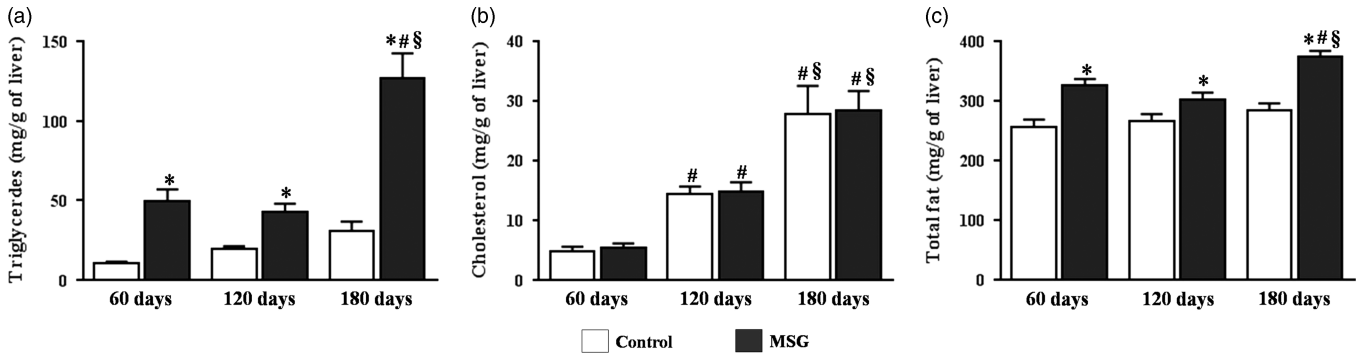
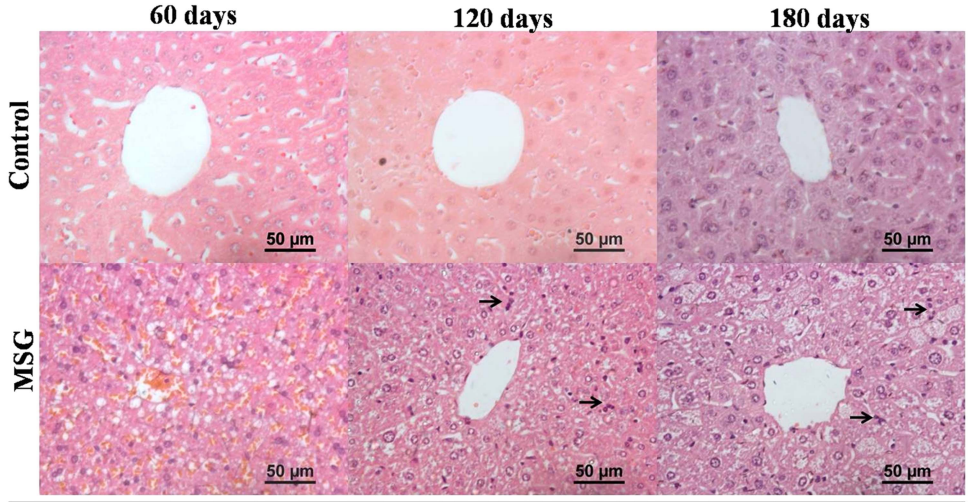


Fig. 2. Hepatic lipid profile. Samples from control and monosodium L-glutamate (MSG) mice livers were taken to assess lipid profile. The concentration of triglycerides (*a*), cholesterol (*b*) and total fat (*c*) in liver were evaluated. $n = 5–7$, mean ± S.E.M., $^{*}P < 0.05$ *v.* Control (at the same age), $^{\#}P < 0.05$ *v.* 60 days (at the same group), $^{\S}P < 0.05$ *v.* 120 days (at the same group). Student's *t*-test for MSG *v.* Control at the same age; ANOVA with Newman-Keuls' post-test for MSG/Control subgroups.



	60 days		120 days		180 days	
	Control	MSG	Control	MSG	Control	MSG
Steatosis	0,00 ± 0,00	1,20 ± 0,37*	0,00 ± 0,00	1,50 ± 0,34*	0,00 ± 0,00	2,17 ± 0,31*#§
Ballooning	0,00 ± 0,00	0,80 ± 0,20*	0,00 ± 0,00	1,33 ± 0,21*#	0,00 ± 0,00	1,50 ± 0,22*#
Inflammation	0,00 ± 0,00	0,21 ± 0,19	0,00 ± 0,00	1,00 ± 0,36*#	0,00 ± 0,00	1,17 ± 0,31*#
Total	0,00 ± 0,00	2,20 ± 0,66 *	0,00 ± 0,00	4,00 ± 0,68*#	0,00 ± 0,00	4,83 ± 0,60*#

Fig. 3. Hepatic histopathology and non-alcoholic fatty liver disease (NAFLD) activity score. Histopathology from control and MSG mice livers was analyzed and NAFLD activity score was applied. Black arrows indicate infiltration foci of polymorphonuclear cells. 400× magnification, H&E stain. $n = 5–7$, mean ± S.E.M., $^{*}P < 0.05$ *v.* Control (at the same age), $^{\#}P < 0.05$ *v.* 60 days old (at the same group), $^{\S}P < 0.05$ *v.* 120 days (at the same group). Student's *t*-test for MSG *v.* Control at the same age; ANOVA with Newman-Keuls' post-test for MSG/Control subgroups.

was observed nor in MSG_{120} neither in MSG_{180} (Fig. 4c and 4d, respectively). Despite the inflammatory condition of the liver, no difference has been found for serum cytokines concentration between the groups at any age (Fig. 5). Multivariate analysis was performed to further assess which morphometric and/or metabolic features were correlated to total NAS in each group. R^2 value for this analysis was >70%, which showed that RFP was the only variable to independently correlate with NAFLD-to-NASH progression in MSG-obese mice (Table 2).

Discussion

There are many genetic and dietetic murine models for NAFLD study available in literature, but a few display inflammation-associated progression to NASH or histopathological features resembling human disease.[28] In the present study, we showed that MSG obese mice precociously develop NAFLD, which promptly progressed to NASH, following a time-course consistent with the Two Hits Hypothesis.[3] Our MSG mice exhibited steatosis at the young age of 60 days old, evolving into inflammation at adulthood (120 and 180 days old), opposing other studies that showed NAFLD onset to occur at older ages in this animal model.[17,23]

Hypothalamic damage consequent from neonatal administration of MSG induces vagus nerve hyperactivity[29] leading to precocious hyperinsulinemia,[30] which not necessarily concurs with hyperglycemia.[14,31] MSG_{60} mice showed fasting serum glucose levels lower than CTR_{60}, suggesting higher peripheral glucose uptake by insulin-sensitive tissues. On the other hand, MSG_{60} showed nearly two-fold higher fasting serum TG levels, a finding consistent with increased triglyceride exportation from liver. In a previous report, we showed that hypertriglyceridemia displayed by MSG obese rats was possibly correlated with decreased hepatic insulin sensitivity, as the protein expression of the hepatic isoform of microsomal triglyceride-transfer protein,

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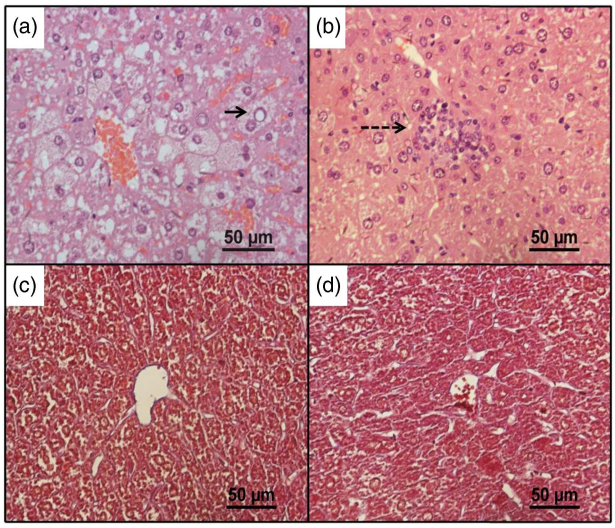

Fig. 4. Non-alcoholic steatohepatitis (NASH) lesions and fibrosis absence. Additional lesions were detected on livers from MSG_{180}, such as glycogenated nuclei (*a*), indicated by black arrow and microgranulomes (*b*), indicated by dashed arrow; H&E stain. Masson's Trichrome stain for livers of MSG_{120} (*c*) and MSG_{180} (*d*). 400× magnification.

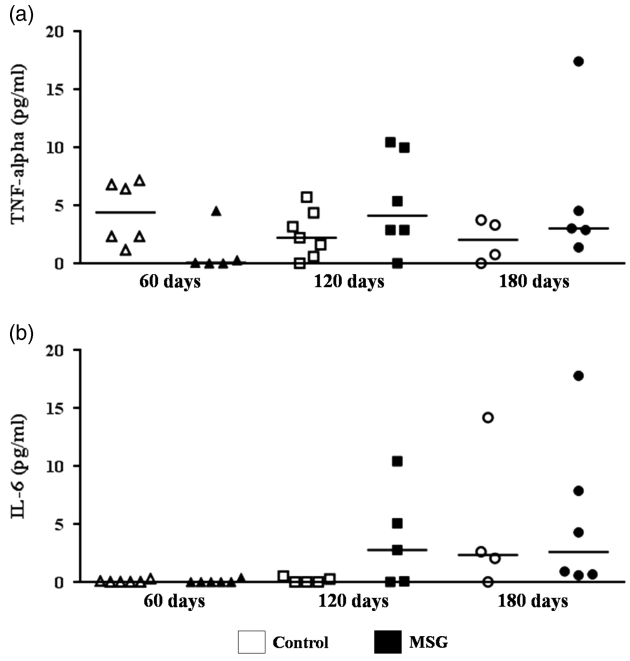

Fig. 5. Serum cytokines by flow cytometry. TNF-α (*a*) and IL-6 (*b*) concentrations were assessed in serum samples by flow cytometry. $n = 5$–7, median, Student's *t*-testfor MSG *v*. Control at the same age; ANOVA with Newman-Keuls' post-test for MSG/Control subgroups.

an enzyme responsible for the insulin-dependent assembly and secretion of very-low density lipoprotein (VLDL) particles,[21] was increased. Assessment of TyG Index in our 60-day-old animals did not show difference between MSG and CTR, suggesting a still preserved peripheral insulin sensitivity in MSG_{60}. However, given the established relationship between hepatic insulin resistance and increased secretion of VLDL particles,[32] these results allow us to suggest that increased serum TG levels found in MSG_{60} resulted from impaired hepatic insulin sensitivity, which might occur before the onset of peripheral insulin resistance caused by the well-known hyperinsulinemia of MSG model.[14]

Table 2. Multivariate analysis for cumulative NAS value

	OR	*P*-value	95% CI
Age	0.998	0.801	0.982–1.014
Group	25.753	0.16	0.249–2658.822
Serum triglycerides	1.013	0.25	0.990–1.038
Fasting glucose	1.008	0.319	0.991–1.026
RFP	16.948	0.017	1.736–165.500
PFP	0.951	0.914	0.368–2.461
Lee Index	0.95	0.085	0.892–1.008
TyG value	0.38	0.309	0.056–2.605
Hepatic triglycerides	1.011	0.449	0.981–1.043
Total fat in liver	0.999	0.947	0.980–1.020

RFP = retroperitoneal fat pad; PFP = periepydidimal fat pad.
R^2 value = 71.43%.

MSG_{60} exhibited elevated fat liver accumulation, mainly characterized by increased TG content inside hepatocytes, which resulted in microvesicular steatosis on zone 3. Even though we have not assessed lipogenic pathways in this study, it is possible to suggest that such lipid accumulation is associated to the triggering of unfolded hepatic protein response, as previously shown,[21] which also relates to precocious hepatic insulin resistance in the physiopathology of NAFLD.[33] This hepatic condition is known to predispose individuals to T2DM.[34] Indeed, MSG_{120} developed T2DM, with fasting hyperglycemia and peripheral insulin resistance, confirming previous reports in which MSG mice were diabetic around 10 weeks of age.[18]

Hepatic TG deposit enhancement clearly relates to NAFLD onset in MSG_{60} and steatosis progression in following ages. In human NAFLD patients, it is described that 59% of TG into hepatocytes accrue from circulating lipids, whereas 26% comes from *de novo* lipogenesis.[35] Hepatic d*e novo* lipogenesis is increased in *in vitro* hyperinsulinemia.[34] In humans, hyperinsulinemia has been shown to be independently associated to NAFLD onset, despite of glycemic status,[36] as 'pre-diabetes' has been appointed as a precursor of NAFLD.[34] Besides, it has also been shown that free fatty acids regulate hepatic TG synthesis through an insulin-independent pathway,[37] which might also contribute for this precocious NAFLD development.

At 120 days old, MSG mice were hyperglycemic, meeting the criteria for MetS, as well as showing increased TyG Index, which support the development of peripheral insulin resistance. In humans, TyG Index has been validated as a reliable tool for insulin resistance assessment[25,38–40] and as a biomarker to identify NAFLD.[41,42] For mice, this is the first time TyG Index is correlated with NAFLD progression, though it has already been applied for peripheral insulin resistance assessment.[43–46] We have not measured serum insulin levels for additional HOMA-IR calculation, even though it is well-documented that MSG obese mice develop hyperinsulinemia since early ages because of increased vagotonia.[29] Besides, MSG mice showed low protein expression of glucose transporter 4 in insulin sensitive tissues, which contributed for glucose intolerance assessed at 12 weeks of age.[47]

Despite MSG_{120} not having an increase in hepatic total fat content in relation to MSG_{60}, hyperglycemia and peripheral

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insulin resistance onset can explain NAFLD progression, as some studies have correlated T2DM with NAFLD severity.[34] To the best of our knowledge, the younger age formerly describing NASH occurrence in MSG-treated mice was at 20 weeks old in Swiss[48] or 18 weeks old in C57BL/6J[49] strains. Therefore, the period between 60 and 120 days comprises crucial events, such as liver inflammation and peripheral insulin resistance, to comprehend this progression. MSG_{180} essentially exhibited the same scenario as MSG_{120}, but higher hepatic TG storage, as well as larger steatosis and eventual microgranulomes and glycogenated nuclei, markers of hepatocyte injury.[50] Corroborating our study, previous reports have shown that MSG-obese mice liver predominantly exhibits microvesicular steatosis,[17,23] which constitutes a feature of severity.[50] Noteworthy, none of our mice acquired liver fibrosis. Similar to humans,[51] fibrosis seems to be a mid-to-long term event in MSG mice non-alcoholic fatty liver progression, as mild fibrosis in this model has been described only over 48 weeks old of age.[17,23]

We next sought to identify what variables were independently associated to the NAS total value on MSG-obese mice. Among all variables assessed by multivariate analysis, RFP was the only to independently correlate with NAS value. RFP is a rodent fat depot whose metabolic role is comparable with human visceral adipose tissue.[52] Thus, likewise human NAFLD,[53] our data support that visceral adipose tissue is an independent risk factor for NAFLD onset and progression in MSG mice. Despite the well-documented relationship between visceral adipose tissue accumulation and decreased peripheral insulin sensitivity,[54] RFP lipectomy did not improve glucose tolerance in mice.[55] Contrariwise, increased visceral lipolysis may lead to fat liver accumulation,[56] suggesting the involvement of insulin-independent lipogenic mechanisms on NAFLD pathophysiology.

According to the multiple hits hypothesis, endocrine, immunological and even intestinal factors might gather to promote (or not) the inflammation typically associated to NAFLD-to-NASH progression.[57] In spite of that, our MSG mice did not display altered serum cytokine levels as compared with lean controls, corroborating a previous study which showed that 6-month-old MSG mice had higher hepatic but unaltered serum levels of TNF-α and IL-6.[58] Indeed, MSG rodents have hypercorticosteronemia,[59] which suppresses systemic pro-inflammatory cytokines production.[60] However, MSG rodents also have resistance to corticosterone in adipose tissue,[61] and corticosterone excess can induce oxidative stress[62] and lipid accumulation[63] in hepatocytes, which might explain the paradoxical inflammation upon these tissues. We did not assess hepatic cytokines gene or protein levels, constituting a limitation of our study. However, hepatic inflammatory infiltration found in MSG_{120} and MSG_{180} mice allows us to suggest an increased production of pro-inflammatory cytokines in MSG hepatic microenvironment. Interestingly, Yamazaki *et al.* reported the absence of hepatic neutrophil infiltration, but elevated TNF-α gene expression in the liver of 16-week-old MSG mice.[64]

Taken together, our data show that MSG mice display precocious onset of fat liver accumulation and that the period between 60 and 120 days of life comprises a crucial window of physiopathological events involved in NAFLD-to-NASH progression. In this way, our study contributes to the usage of MSG obese model as a tool for future investigations on early molecular mechanisms underlying NAFLD and its mid-to-long term progression toward advanced fatty liver diseases, particularly in the context of early hyperinsulinemia.

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Conflicts of Interest. None.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the Brazilian National Council for Animal Research Care (CONCEA) and has been approved by Committee for Animal Care and Welfare (CEUA) of the Federal University of Maranhão, ruling no. 001/2009.

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