ISSN: 2640-7477

Research Article

Journal of Gastroenterology & Digestive Systems

Fast Induction of Hcc in Nash Rodent Model: A Simple Diet Associated with A Chemical Carcinogen

Carolina Antunes Marques[1], Ana Beatriz Souza de Oliveira[2], Cinthia Laureano Pavan[2], Claudia P Oliveira[3], Ilka de Fátima Santana Ferreira Boin[4], Bruno Cogliati[5], Dalísio Santi Neto[6], Rita de Cassia Martins Alves da Silva[7], Renato Ferreira da Silva[7]

[1]*Bolsista PIBIC/CNPq*

[2]*Discentes da Faculdade de Medicina de São José do Rio Preto- FAMERP*

[3]*Departamento de Gastroenterologia da Faculdade de Medicina da Universidade de São Paulo- USP*

[4]*Departamento de Cirurgia da FCM - Universidade Estadual de Campinas- UNICAMP*

[5]*Laboratório de Patologia Morfológica e Molecular (LAPMOL) da Faculdade de Medicina Veterinária da Universidade de São Paulo – FMV-USP*

[6]*Departamento de Patologia do Hospital de Base de São José do Rio Preto - FAMERP*

[7]*Unidade de Transplante de Fígado do Hospital de Base- FAMERP*

***Corresponding author**

Renato Renato Ferreira da Silva, Department of Surgery, Faculty of Medicine, São José do Rio Preto, São Paulo, Brazil

Submitted: 04 Sep 2020; **Accepted:** 10 Sep 2020; **Published:** 15 Sep 2020

Citation: *Carolina Antunes Marques, Ana Beatriz Souza de Oliveira, Cinthia Laureano Pavan, Claudia P Oliveira, Ilka de Fátima Santana Ferreira Boin, Bruno Cogliati, Dalísio Santi Neto, Rita de Cassia Martins Alves da Silva, Renato Ferreira da Silva (2020) Fast Induction of Hcc in Nash Rodent Model: A Simple Diet Associated with A Chemical Carcinogen J Gastro & Digestive Systems 4(2): 31-37.*

Abstract

Introduction: *Non-alcoholic fatty liver disease (NAFLD) have been increasing as an important cause of hepatocellular carcinoma (HCC). Experimental models are crucial to identify some pathways in the pathogenesis of HCC secondary to NAFLD.*

Objective: *To systematize an experimental hybrid rodent model of HCC secondary to NAFLD.*

Material and Methods: *Fourteen male Sprague-Dawley rats, weighting 350-500g, were fed with choline-deficient high-fat diet and diethylnitrosamine (DEN) in the drinking water for 16 weeks. The animals were separated into two groups: 7 received DEN in water (100mg/ml) associated to choline-deficient high-fat diet and 7 received only choline-deficient high-fat diet. Histological and immunohistochemical analysis were also performed.*

Results: *All animals had definitive diagnosis of NASH in both groups with hepatocellular ballooning, steatosis and inflammation. In the group that received high-fat choline deficient diet, only two animals had micro nodular cirrhosis. However, in DEN group with high-fat choline deficient diet, all animals had gross lesions, major nodulations and fibrosis, visible in macroscopy. Besides, according of Edmondson-Steiner classification, 71% of the animals that received DEN + Diet with positive CK19 and oval cells and developed some grades of dysplastic and tumoral lesions. Moreover, in this model we could identify beyond NASH progression, some tumor-development stages, which means this model shows different treatment and study targets.*

Conclusion: *The model is accomplishable, promotes fast induction of HCC in NAFLD context without much complexity to be executed and will be implemented as an interesting model to study new drugs to HCC secondary a NASH.*

Keywords: Hcc; Hepatocellular Carcinoma; Cancer; Liver; Rats; Animal Model; Sprague-Dawley.

Introduction

Nonalcoholic fatty liver disease (NAFLD) a large spectrum of the disease since simple steatosis to steatohepatitis that include beyond stestosis, hepatocellular ballooning, inflammation and some stages of fibrosis and can progress to cirrhosis and hepatocellular carcinoma (HCC). NAFLD is currently the most prevalent chronic liver disease in western countries and its incidence has increased dramatically in recent years in both developed and underdeveloped countries due to the increasing rates of obesity in the world population [1].

Liver cancer is the second leading cause of cancer-related death in the world, with HCC being the most frequent type of liver cancer (2). Currently, NAFLD is the fastest growing cause of HCC in the population, parallel to the obesity and type 2 diabetes (3). The development of HCC is directly linked to obesity and type 2 diabetes, which are independent risk factors for the development of the disease and play an essential role in its pathogenesis (4). Several factors also involved in the genesis of NAFLD participate in the pathogenesis of HCC. IR and AGL accumulation, which lead to oxidative stress and inflammation, participate in this chain for generating lipid peroxidation products capable of leading to mutations in the p53 gene (5).

It is known that many factors involved in the genesis of NASH are implicated in the development of HCC. However, current animal models are still not completely adequate to understand and simulate the pathogenesis of CHC secondary to NASH NAFLD, as a consequence of a set of metabolic disorders, presents itself as a complex disease with difficulty to be mimicked at the experimental level. Thus, the development of experimental models is extremely important to understand the pathogenesis of HCC secondary to NAFLD and to analyze the interference of drugs in the natural course of the disease.

Several animal models have been developed to mimic the genesis of HCC in humans and facilitate the study of the pathogenesis of this disease (HCC derived from NASH). These models include hypercaloric diet, diethylnitrosamine (DEN) as a carcinogen, in different routes of application, as well as the choline-deficient diet, fructose and CCl4 (6) but none mimics it contemplating all its facets and peculiarities. In addition, the complexity of some models, cost and extended execution time make them difficult to apply.

Material and Methods

Study Design

Fourteen male Sprague-Dawley rats, weighting 358-504 g, were housed in the animal facility of the Clinical and Experimental Center of Hospital de Base\FAMERP. The animals were allocated in a room with temperature, humidity and controlled ventilation, in a 12-hour cycle of light and shade, without restriction of water and feed, being always handled with the use of gloves and a mask and changing shavings 3 times a week. All work was carried out according to the specifications of the ethics committee on the use of animals (CEUA) of Hospital de Base/FAMERP approved under protocol number 001-002275 / 2017. These animals were separated into two groups of 7 animals: 1) NASH group- NASH was induced in animals by a choline-deficient diet, with a high concentration of fat (35% total fat, 54% trans-fat, acquired from Rhoster Ltda, Brazil) for 16 weeks; 2) NASH-HCC group- in addition to the same diet, it was supplied to animals diethyl nitrosamine (DEN) (IUPAC: nitrosodiethylamine, Sigma Chemical) in drinking water at a concentration of 100mg / l for 16 weeks as previously described (7). At the end of the period, the animals were euthanized in the laboratory and submitted to necropsy. Before the procedure, the animals were anesthetized intraperitoneally with ketamine (Cristalia, Brazil) dose of 80 mg/kg and xylazine (Bayer, Brazil) dose of 10 mg/kg. Nodules ≥0.2 cm were considered.

Liver Biopsy and Histology

Histological evaluation was carried out by two institutions: 1) immunohistochemistry was performed with cytokeratin 19 (CK19) as an oval cell marker and lesion classification according to Edmondson-Steiner (1954) (8), in the pathology laboratory of Hospital de Base/FAMERP under the responsibility of Prof. Dalisio Santi Neto, (DSN). Histology analysis was done by Prof. Dr. Bruno Cogliati (BG), veterinary pathologist at the Faculty of Veterinary Medicine of USP in São Paulo, who performed the evaluation of the slides in HE.

Immediately after the animals were euthanized, fragments of the liver tissue were fixed in buffered formaldehyde (pH 7.2-7.4) for 24 hours and, later, processed and embedded in paraffin according to standard procedures. Histological sections of 5 µm were stained by hematoxylin-eosin (HE) techniques for histopathological evaluation. NASH were graded according to the criteria established by Kleiner et al. (2005), with minor modifications, generating the activity index of non-alcoholic fatty liver disease (NAS) (9). This index includes only characteristics of the active lesion, being defined by the sum of the degrees of macro and / or microgoticular steatosis (0 to 3), lobular inflammation (0 to 3) and ballooning (0 to 2). Thus, the values of the NAS index vary between 0 and 8, with NAS ≥ 5 correlating with the diagnosis of non-alcoholic steatohepatitis (NASH), while NAS <3 is not considered NASH. On the other hand, animals with NAS between 3 or 4 can be considered as probable carriers of NASH. Proliferative and neoplastic lesions were classified according to Thoolen et al. (2010) (10).

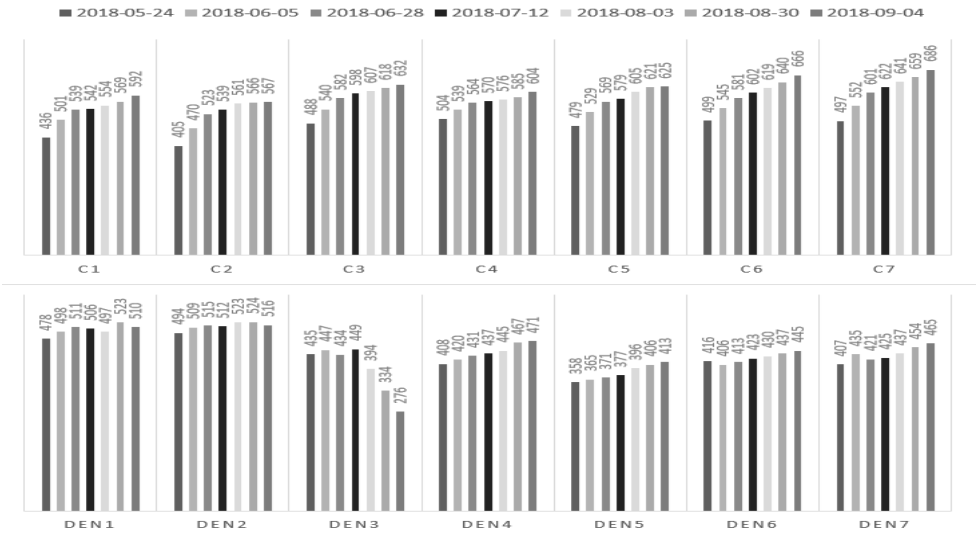
Immunohistochemistry analysis was performed utilizing by formalin-fixed paraffin- embedded tissues sectioned at 5 µm. The heat-induced epitope retrieval was performed in an electric pressure cooker (110V, 60Hz) for 15 mins, using citrate (pH 6.0) buffer. After blocking endogenous peroxidase with 6% H2O2 solution (Merck, USA) for 30 min, the slides were incubated in a humidified chamber overnight at 4°C with the rabbit primary anti-Hep-Par1 (Dako, 1:500), cytokeratin 19 (CK19, Leica Novocastra,

1:200) e glutamine-synthetase (GS, Millipore, 1:3000). HCC was considered for nodules with positive results for GS or HEP-PAR-1, and negative for CK-19 [Figure 1]. Antibody dilution was CK-19 1:200.

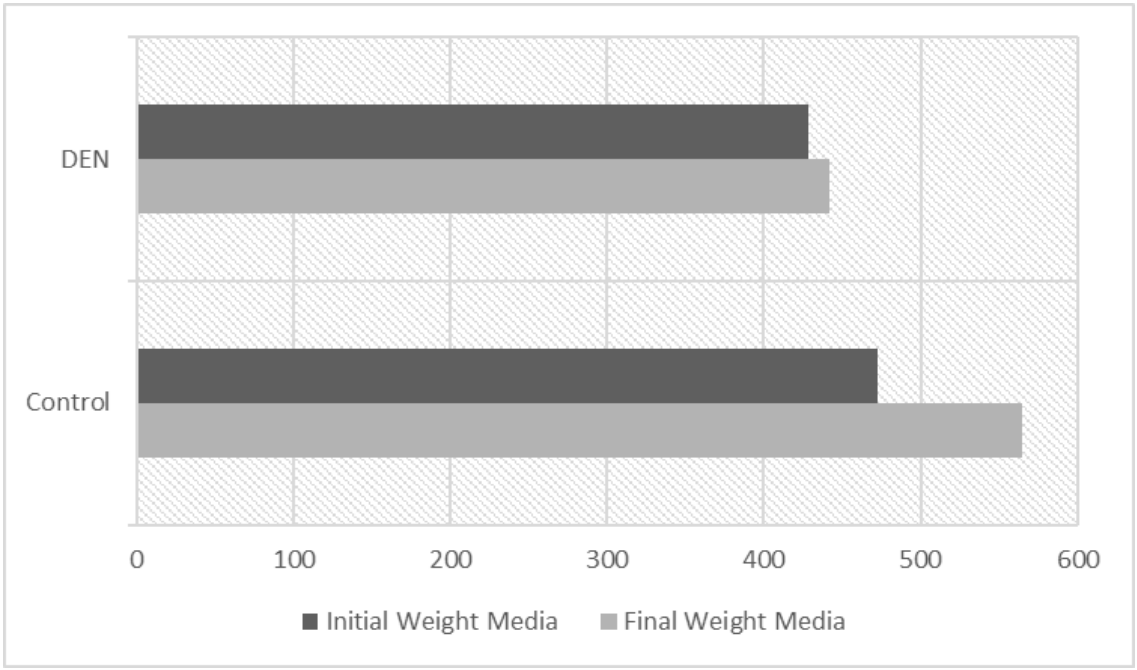
For statistical analysis, values of mean, median and standard deviation were calculated according to the distribution pattern of variables using the software Graph Pad Prism version 7.

Results

All animals completed the 16 weeks of experimentation. During the 16 weeks, the animals were monitored daily and we observed increase of weight throughout the study, except for one animal in the DEN group (identified as E3) which started with progressive weight gain, but on the 39th day of induction presented a nodule in the facial region corresponding an ulcerated facial abscess, started to lose weight (Graph 1).



Graph 1: Animal weight during the period in grams (g) – DEN: animals that received diethyl nitrosamine and special diet. C: group which received only the special diet.



Graph 2: Group weight media in grams (g). DEN: animals that received diethylnitrosamine and special diet. Control: group which received only the special diet. SD: Standard deviation.

Upon laparotomy, at macroscopy, all animals had steatosis in both groups. In the NASH group that received only high-fat choline deficient diet, only two animals had in visible form, granulations in only one liver segment suggestive of cirrhosis. The others had enlarged and yellowish appearance of fatty liver (Figure 1A). In the NASH-HCC group which the animals received DEN, all had gross lesions, major nodulations and fibrosis, visible in macroscopy (Figure 1B). Nodes strongly suggestive of HCC were seen in 5 of the 7 animals (about 71%). No animal had significant ascites. The lungs of animals that received DEN had nodules that were removed for histological analysis.

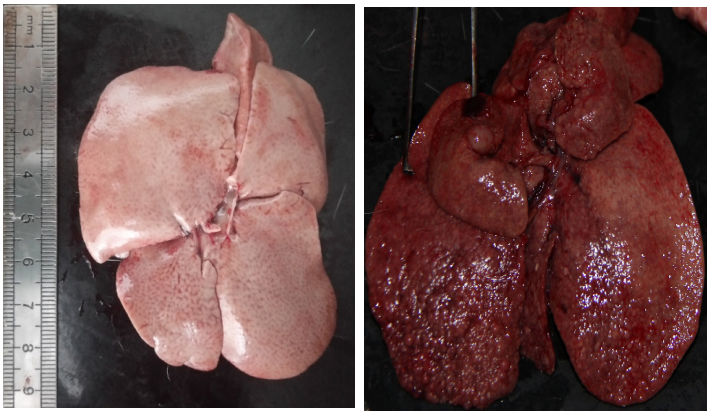


Figure 1A: Macroscopic fatty liver from NASH group;
Figure 1B: Macroscopic fatty liver with micronodular aspect in NASH-HCC group

Under light microscopy, all animals had NASH with NAS ≥ according Kleiner et al (Table 1). In the evaluation performed at the Hospital de Base \ FAMERP, of the animals that received diethyl nitrosamine, 5 (five-71%) presented lesions suggestive of extensive and poorly differentiated HCC (grades III and IV), classified according to Edmondson-Steiner (8), ranging from 1 to 3 focus of HCC in each animal, with positive CK19 and oval cells. Two developed in addition to HCC, focal hemangioma. Two did not develop any type of tumor, the latter two being negative for CK19 (Table 2). Nevertheless, in the evaluation utilizing a specific classification to proliferative and non-proliferative lesions of the rat and mouse hepatobiliary system (10) by the veterinary pathologist (USP) diagnosed only in 3 animals HCC and three others developed dysplasias (Table 3).

In all animals, pulmonary histology showed extensive bronchopneumonia, with abscesses. The facial lesion that one of the animals developed was typical of squamous cell carcinoma, which can be attributed to a tumor of the Zymbal gland associated with the use of DEN.

Table 1: Histopathological analysis and activity index of non-alcoholic fatty liver disease

Rats	Hepatocellular balloning (0-2)	Steatosis (0-3)	Lobular inflammation (0-3)	NAS (0-8)
NASH 1	1	3	1	5
NASH 2	2	3	1	6
NASH 3	1	3	1	5
NASH 4	1	3	1	5
NASH 5	1	3	1	5
NASH 6	1	3	1	5
NASH 7	1	3	1	5
NASH-DEN 1	2	3	2	7
NASH-DEN 2	2	3	1	6
NASH-DEN 3	1	3	3	7
NASH-DEN 4	2	2	2	6
NASH-DEN 5	1	3	2	6
NASH-DEN 6	2	3	2	7
NASH-DEN 7	1	3	2	6

NAS: nonalcoholic fatty liver disease activity score (NAS)

Table 2: Histopathological evaluation to neoplastic lesions in animals with DEN performed according Edmonson & Steiner (ES) Classification and Immunohistochemistry by CK 19.

Rats	HCC	CK19
NASH-DEN 1	Absent	Negative
NASH-DEN 2	HCC – 2 lesions ES 3	Oval cells positive
NASH-DEN 3	HCC – 1 lesions ES 3-4	Oval cells positive
NASH-DEN 4	HCC – 3 lesions ES 3-4	Oval cells positive
NASH-DEN 5	HCC– 3 lesions ES 3-4 Focal Hemangioma	Oval cells positive
NASH-DEN 6	Absent	Negative
NASH-DEN 7	HCC – 1 lesions ES 2-3 Focal Hemangioma	Oval cells positive

ES: Edmondson-Steiner Classification, 1954

Table 3: Histopathological evaluation to proliferative and neoplastic lesions in animals with DEN performed utilizing a specific classification to rat and mouse hepatobiliary system (Thoolen et al 2010).

Rats		Other proliferative lesions	Other lesions
NASH-DEN 1	Absent	-Presence of several foci of dysplastic hepatocytes. - hepatoblastoma (single lesion composed of basophilic, pleomorphic cells and with a high degree of typical mitosis).	- Hepatocyte megalocytosis. - Hepatical cirrhosis. - Lung: suppurative broncho-pneumonia
NASH-DEN 2	HCC with pleomorphic cells, evident nucleoli, pseudoglandular pattern and with mixed intratumoral inflammatory infiltrate.	- Presence of regenerative nodules. - Presence of several foci of dysplastic hepatocytes and cystic bile ducts	q
NASH-DEN 3	HCC with pleomorphic cells and central necrosis area	- Presence of several foci of dysplastic hepatocytes. - Proliferation of oval cells.	- Hepatocyte megalocytosis. - Hepatic cirrhosis. - Lung: suppurative broncho-pneumonia
NASH-DEN 4	HCC with pleomorphic, basophilic cells, with a high mitotic index and central bleeding area.	.-Presence of a hepatocellular adenoma, bounded by a fibrous, eosinophilic capsule and with intratumoral lymphoplasmocytic inflammatory infiltrate	- Hepatocyte megalocytosis. - Hepatic cirrhosis. - Lung: suppurative broncho-pneumonia
NASH-DEN 5	Absent	- Two focal hemangiomas - Presence of several foci of dysplastic hepatocytes.	- Hepatocyte megalocytosis. - Lung: suppurative broncho-pneumonia
NASH-DEN 6	Absent	- Presence of several foci of dysplastic hepatocytes.	- Hepatocyte megalocytosis. - Hepatic cirrhosis. - Lung: suppurative broncho-pneumonia
NASH-DEN 7	Absent	-A focal lesion compatible with hemangioma.	- Hepatocyte megalocytosis. - Lung: suppurative broncho-pneumonia

Discussion

This experimental model is feasible to be implemented for the development of HCC in liver with steatosis, it is advantageous to fear that it will not be long term and mimetize a HCC NASH related. This model was described previously by Lima et al, (7). however, in the current study we reduced the DEN concentration (100µg/dayly) and we found the presence of advanced fibrosis or cirrhosis in all animals, as well as dysplastic nodules and advanced HCC different from the previously described model with higher doses of diethyl nitrosamine (7). These findings highlight the clinical relevance of our model, as most preclinical studies used younger animals with less advanced disease, thus not reflecting the hepatic microenvironment observed in humans (11) . Besides, in 2019 Zhong published a review of experimental models of HCC in NASH, a model that encompassed the entire pathogenesis of non-alcoholic fatty liver disease was not identified, all of which have their importance in elucidating specific aspects, but none are exempt from failures (6). In addition, this present model could mimic all the steps of the development of HCC secondary to NASH in a short time compared to others (6).

Another interesting aspect in the present study is discuss about the histologic classification used in animal models. In the histological analysis there was a difference in results from the point of view of the pathologists, with 3 of the 7 animals using DEN and diet developing cancer according to the classification of the veterinary pathologist (42.85%) by Thoolen et al classification which is used in rats and mouse, but another three developed dysplasia that are pre-malignant lesions. However, utilizing human classification as Edmonson & Steiner it was observed in 71.42% of the animals HCC microscopically confirmed by CK19 (oval cells positive) in this model, this model was able to provide a question about intra and intertumor variability according HCC classification used in animal's models and evoke new studies to better classifications in these rodents.

Costa et al (12) recently demonstrated in similar study that despite treatment with sorafenib in this model, the mortality rate (60%) was similar in group that use sorafenib or not, resulting from the severity of liver cirrhosis and advanced HCC; sometimes with decompensation in ascites (about 10% in both groups) and pulmonary metastasis (12) . An important thing is that we did not loss animals with smaller concentration of DEN and we obtained a sampling of the disease at different stages of development. However, it is necessary to say that this is an aggressive model, and that animals can develop neoplastic lesions other than HCC.

It has already been described that the model used in this work induces the development of cancer derived from oval cells (7). Oval cells are the primordial cells of the liver, which can differentiate into hepatocytes or cholangiocytes. They are activated in the presence of chronic liver injury, and are related to a worse prognosis when HCC is derived from this strain compared to HCC derived from hepatocyte (7,13). The mechanism by which the disease develops in these animals involves an increase in oxidative damage (due to the increased metabolism of a diet enriched with fat) in association with a carcinogen with a known mechanism of action (7). By this experimental model, it was possible to observe the development of steatosis and grade 4 hepatic fibrosis in all studied samples, proving the development of NAFLD. Besides, the NAS of all samples was ≥5, evidencing the presence of NASH. The majority of studied nodules were classified in grade III or IV of Edmondson & Steiner's classification, representing poorly differentiated tumors, which tend to have worse prognosis and to determinate more advanced disease...

Others animal models of HCC secondary to NASH have been described as a induced with high- fat/fructose diet and sedentary lifestyle which was demonstrated the NASH development at six months of experimentation and the HCC development at 12 months in only 60% of animals (14). This result shows that is possible to induce HCC experimentally in a model that simulates the majority of NAFLD patient's lifestyle. However, there is a difficulty in the reproducibility of this kind of model because of the longtime of experimentation needed to tumor development. Other studies also took a long time to demonstrate the development of HCC and NASH. Two models were developed with C57BL/6J rats, genetically predisposed, after high-fat diet consume. The study DIAMOND used high-fat diet in association to ad libitum consume of glucose and fructose and developed in 89% of the studied rats HCC between 32 and 52 weeks of experimentation (15). It is an interesting model because it does not have chemical carcinogenic, however it took a long time to able to be used in preclinical therapeutic studies. More recently, Kawa-Yoshida et al, (16) developed a model used choline-deficient, L-aminoacid-defined, high- fat diet (CDAHFD) developed HCC between 36 and 60 weeks of exposition to this diet.

As for pneumonias, in the last three weeks of induction, the animals had noisy and altered breathing (including those that did not receive DEN) so that the route of infection is questioned, since the animals were always handled with great care, using mask and proper hand hygiene. In addition, all data obtained from contaminated animals must be analyzed with caution. With relatively low cost and ease of execution, this model will be implemented in our institution for therapeutic, genetic and molecular study of HCC and NASH.

In conclusion, the present study was able to developed an animal HCC model secondary a NAFLD in faster time and confirming the HCC diagnosis through histological and immunohistochemically analysis. These results show the applicability of this HCC rodent model in secondary to NAFLD to test new therapeutic approaches.

Financial Support
The study was funded by Prof. Dr. Renato Ferreira da Silva - FAMERP with the help of Profa. Dr. Ilka Boin - UNICAMP. The animals were donated by Profa. Dra. Claudia Oliveira – USP.

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