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Omar Motiño, Sijing Li, Flavia Lambertucci, Gerasimos Anagnostopoulos, Léa Montégut, et al.. A mouse model of hepatocellular carcinoma induced by streptozotocin and high fat diet. *Liver Carcinogenesis*, 2769, Springer US, pp.67-75, 2024, *Methods in Molecular Biology*, 10.1007/978-1-0716-3694-7_5 . hal-04596680

HAL Id: hal-04596680

<https://hal.science/hal-04596680>

Submitted on 31 May 2024

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A mouse model of hepatocellular carcinoma induced by streptozotocin and high fat diet

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Running head: Streptozotocin-high fat diet induced mouse model of HCC

Abstract

Hepatocellular carcinoma (HCC) is the most common type of liver cancer and the second most common cause of cancer-related death. HCC is associated to chronic diseases such as viral hepatitis, alcoholic and non-alcoholic fatty liver disease (NAFLD), diabetes mellitus and obesity, among others. Although pre-clinical models have been investigated to mimic the transition from NAFLD to HCC, they do not accurately reproduce the phenotypic evolution from simple steatosis to steatohepatitis, fibrosis/cirrhosis and HCC. Hence, these models have failed to demonstrate the influence of diabetes on hepatic carcinogenesis. Here, we report a novel mouse model of HCC triggered by fast-developing diabetes and NAFLD. The first step consists in a single intraperitoneal injection of a low dose of streptozotocin into neonatal C57BL/6J mice to induce type 2 diabetes. In a second step, mice are fed with high-fat diet to accelerate the development of simple steatosis. Continuous high-fat diet exacerbates hepatic fat deposition with increased lobular inflammation (by activation of foam cell-like macrophages) and fibrosis (by activating hepatic stellate cells), two representative pathological traits of steatohepatitis/fibrosis. After 20 weeks, all mice developed multiple HCCs. This model of hepatic carcinogenesis triggered by diabetes mellitus and NAFLD offers the advantage of being rapid and accurately recapitulates the pathogenesis of human HCC without the need of administering hepatic carcinogens.

Keywords

Hepatocellular carcinoma, non-alcoholic fatty liver disease, diabetes mellitus, non-alcoholic steatohepatitis, streptozotocin, high-fat diet.

1. Introduction

Liver cancer is the second cause of cancer-related death, behind lung cancer, with a 5-year survival rate (in the range of 5-30%) **(1, 2)**. There are several types of primary liver cancer, where hepatocellular carcinoma (HCC) is the most common type of liver malignancies. Human hepatocarcinogenesis is typically related to chronic diseases: viral hepatitis, alcoholic fatty disease (AFLD), obesity/metabolic syndrome (MS), diabetes mellitus (DM), and non-alcoholic fatty liver disease (NAFLD), among others **(3)**.

NAFLD is a clinical term to define the pathology that develops in the absence of alcohol abuse and includes a spectrum of alterations ranging from the simple accumulation of fat in the liver, also called non-alcoholic steatosis (NAS), to non-alcoholic steatohepatitis (NASH), and fibrosis, which can ultimately lead to cirrhosis and HCC **(4)**. NAFLD is a major health problem that affects a one-third of adults and also increasingly affects children in developed countries. **(5)**. About 90% of patients with NAFLD have at least one characteristic of MS and about 33% have the full diagnosis **(5, 6)**. For this reason, NAFLD is considered the hepatic manifestation of MS, which includes characteristics such as obesity, DM, insulin resistance, dyslipidemia and hypertension **(7)**. The basis of HCC pathogenesis still remains elusive and therapeutic options are limited to lifestyle interventions (diet and physical activity) **(8)**.

Despite the proven epidemiological links between DM and HCC, there are no suitable mouse models demonstrating HCC acceleration by DM **(9)**. Regarding

animal models of NASH, they can be divided generally into 3 subtypes: genetic, hepatotoxic, and dietary, the latter often usually involving a methionine-choline-deficient diet (MCD) or long-term high-fat diet (HFD) **(10)**. Despite their multiplicity, none of these models reproduce the complete physio-pathological evolution from NAS to NASH, fibrosis and HCC in the context of diabetes **(9)**.

Here, we report a DM/NASH-HCC model that is induced by a combination of chemical and dietary interventions and closely recapitulates the histopathological events occurring in the human liver, and in particular the rapid and stepwise progression from steatosis to NASH and fibrosis **(Figure 1)**. The first step consists in a single intraperitoneal injection with low dose of streptozotocin into neonatal C57BL/6J mice to produce mild islet inflammation and partial destruction. To verify if mice develop DM, the levels of glucose in plasma are analyzed with a glucometer weekly for 4 weeks. Animals with DM show polydipsia and polyuria **(11)**. Regarding this, the cages of the animals should be revised twice per week. In a second step, 4 weeks after birth, streptozotocin-primed mice are fed with HFD to trigger the sequential histological manifestation of NAS, NASH/fibrosis and HCC. NAS and NASH appear as an underlying consequence of the continuous accumulation of fat and the infiltration of macrophages after 5 and 7 weeks, respectively **(10)**. The chronic inflammatory environment promotes the activation of hepatic stellate cells, inducing fibrosis **(12)**. Later, mice develop adenomas after 12 weeks and HCC after 20 weeks. Tumor growth can be monitored by echography for

16 to 20 weeks after initiation of HFD (**Figure 2A**). The development of the tumor depends on the mouse strain used, but close to 100 % of C57BL/6 mice develop HCC. Finally, mice are euthanized after 20 weeks of HFD, and livers are collected and photographed to confirm the presence of macroscopic tumors (**Figure 2B**). To validate HCC diagnosis, histological analysis from the livers can also be performed by hematoxylin eosin saffron (HES) staining. Additionally, the HCC can be characterized by immunohistochemistry, for instance to measure the levels of proliferation marker Ki67 and the expression of the HCC marker alpha fetoprotein (AFP) (**13, 14**). Hepatic damage can also be indirectly assessed by measuring plasma levels of alanine and aspartate transaminases activity (ALT and AST, respectively) (**15**).

2. Materials

2.1. Reagents

1. 5% Glucose.
2. Glucose test strips.
3. High-fat diet (U8978 Version 19, Safe diets).
4. 1M Hydrochloric acid (HCl).
5. Isoflurane (anesthetic gas).
6. Neonatal C57Bl/6J male mice.
7. Ophthalmic liquid gel.
8. Oxygen tank.
9. Regular chow diet (A04, Safe diets).
10. 50 mM Sodium citrate buffer, pH 4.5.
11. Electric trimmer and depilatory cream.
12. Streptozotocin (S0130, Sigma Aldrich).
13. 10% Sucrose.

2.2. Equipment

1. 1-ml syringes.
2. 2-ml microcentrifuge tubes.

3. 100 μm Filter.
4. 27-G needles.
5. Blood glucometer.
6. Aluminum foil.
7. Echography in vivo imagen system (Vevo® 3100 from FUJIFILM VisualSonics).
8. Cotton buds.
9. Heparin tube (16443, Sarstedt).
10. Isoflurane vaporizer system.
11. Scale (for measuring mouse weight).
12. Medical tapes.
13. Gauzes.

3. Methods

3.1. Prepare animals-Time 4-6 hours

At least 4-6 hours prior to initiating the experiment, house 2-5 neonatal (2 days old) male mice with mother female mice per cage at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and $55\% \pm 5\%$ (see **Note 1**).

3.2. Fresh streptozotocin buffer preparation -Time 30 min.

1. Prepare 50 mL (final volume) of a 50 mM sodium citrate buffer, pH 4.5.
 - 1.1. Dissolve 535 mg in 40 mL of deionized water.
 - 2.1. Set the pH to 4.5 by slowly adding 1M HCl (see **Note 2**).
 - 3.1. Once pH = 4.5, complete the volume to 50 mL.
 - 4.1. Filter the solution.
2. Weigh 4 mg of streptozotocin into a 2-ml microcentrifuge tube and cover the tubes with aluminum foil (see **Note 3**).

3.3. Diabetes Mellitus induced by streptozotocin-Time 4 weeks

1. Immediately prior to injection, dissolve the streptozotocin in 50 mM sodium citrate buffer (pH 4.5) to a final concentration of 2 mg/ml (see **Notes 3 and 4**).
2. Perform intraperitoneal injection (i.p.; lower side of the abdomen with a 27-gauge needle) of 200 μg contained in 100 μL of streptozotocin buffer into C57BL/6J mice at 2 days of age. Inject an equal volume of citrate buffer (pH 4.5) i.p. into the control group mice (see **Note 5**).

3. Return the mice to their home cages. Provide free access to normal food and 10% sucrose water.
4. Inspect animals every 2h after injection for 8h and every day for at least 1 week (see **Note 6**).
5. After one week, place the animals in a new cage without food to start fasting (fresh bedding is crucial to avoid the risk that food leftovers or feces are eaten during the fasting period).
6. After 4-6 h fasting, measure glycemia from one drop of blood from the tail vein by a blood glucometer. After this, allow food and water *ad libitum* (see **Note 7**).
7. Check mice weight and measure the glycemia (see **Step 3.3.6**) every week for 3 weeks.
8. Euthanize mice with levels of glycemia less than 180-200 mg/dL after 3 weeks.

3.4. NAS, NASH, fibrosis and HCC accelerated by HFD -Time 20 weeks.

1. Remove the control diet (regular chow diet) and feed mice with high fat diet (HFD). Allow the food and water *ad libitum* (see **Note 8**).
2. Follow the weight weekly and glycemia monthly (as described in **Step 3.3.6**).
3. After 16 weeks, anesthetize mice with 2 % isoflurane and follow the developing of the tumors by echography (see **Note 9**):

- 3.1. Turn on the Vevo® 3100 imaging system and set up the heated platform to 38°C.
- 4.1. Anesthetize the animal with 2% isoflurane and 1.5–2 L/min oxygen flux in the anesthesia chamber.
- 5.1. Transfer the mouse to the anesthesia table and fix it with adhesive plaster, maintaining the vaporization of isoflurane/oxygen mix.
- 6.1. Eliminate the abdomen hair with electric trimmer and then with depilatory cream and use wet gauzes for cleaning the observational area.
- 7.1. Put transmission gel in the abdomen region.
- 8.1. Use the B-mode of MX550D transducer from the imaging system in transversal position and take adequate images of the liver (see **Note 10**).
- 9.1. Return the animal to the cage and re-inspect animals after they wake up.
4. Repeat the step 3.4.3. weekly to follow up the tumor growth up to 20 weeks.
5. One day before week 20 with HFD, remove the food and keep in fasting for 16h (see **Note 11**).
6. Measure the glycemia (see **Step 3.3.6**), collect blood from the submandibular vein in a heparin tube and keep it at 4°C.
7. Euthanize all animals by cervical dislocation.
8. Collect and take images of the liver.

4. Notes

1. Most of streptozotocin-induced diabetic mouse studies are carried on male animals because female mice are less sensitive to islet-cell destruction **(16)**. Moreover, we recommend creating group sizes of 12 to 20 due to the morbidity associated with the streptozotocin treatment, although the protocol detailed is designed to minimize variability. The final number of mice should be equal for each group.
2. Be careful to let the solution homogenize and the pH stabilize between each drop of HCl.
3. IMPORTANT: Use one tube for three mice because streptozotocin degrades within 15 to 20 min after dissolving in the citrate buffer.
4. Create one control group of mice with regular chow diet and injection of control citrate buffer (vehicle), and one control group with HFD without the initial streptozotocin injection (vehicle).
5. The streptozotocin solution should be prepared immediately before use and injected within 5 min of dissolution.
6. Although the streptozotocin dose is low, some mice will die soon after receiving streptozotocin because of the release of insulin from the pancreas (rapid and massive β -cell necrosis), causing fatal hypoglycemia during the first 24h **(17)**. Check the levels of glucose in plasma to prevent fatal hypoglycemia, if glucose levels are < 70 mg/dL **(18)**, inject streptozotocin-treated mice i.p. with 0.5 ml of 5% glucose solution **(17)**.

7. If the levels of glucose are < 70 mg/dL, inject streptozotocin-treated mice i.p. with 0.5 ml of 5% glucose solution to prevent fatal hypoglycemia **(18)**.
8. Change the cage bedding at least 2 times per week because diabetes mice drink a lot of water and urinate constantly, resulting in wet litter and damp environment.
9. Physiological parameters of the animals are included in the ultrasound imaging system: blood pressure electrocardiogram, heart and respiratory rate, temperature. The examination time per animal is of 20 min, approximately.
10. To watch several regions of the liver, apply necessary pressure in the abdomen gently.
11. The animals are changed to a new cage at the beginning of fasting to eliminate the risk of food remnants being left.

Conflicts of interest

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders.

Acknowledgement

GK is supported by the Ligue contre le Cancer (équipe labellisée); Agence National de la Recherche (ANR) – Projets blancs; AMMICa US23/CNRS UMS3655; Association pour la recherche sur le cancer (ARC); Cancéropôle Ile-de-France; Fondation pour la Recherche Médicale (FRM); a donation by Elior; Equipex Onco-Pheno-Screen; European Joint Programme on Rare Diseases (EJPRD); Gustave Roussy Odyssey, the European Union Horizon 2020 Projects Oncobiome and CRIMSON (grant agreement No. 101016923); Fondation Carrefour; Institut National du Cancer (INCa); Institut Universitaire de France; LabEx Immuno-Oncology (ANR-18-IDEX-0001); a Cancer Research ASPIRE Award from the Mark Foundation; the RHU Immunolife;

Seerave Foundation; SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); and SIRIC Cancer Research and Personalized Medicine (CARPEM). This study contributes to the IdEx Université de Paris ANR-18-IDEX-0001. SL and HC are supported by the China Scholarship Council (CSC, file n°. 201907060011 and file n° 201908070134 respectively). UN-R is supported by Axudas de apoio á etapa de formación posdoutoral da Xunta de Galicia – GAIN. N°Expediente: IN606B-2021/015.

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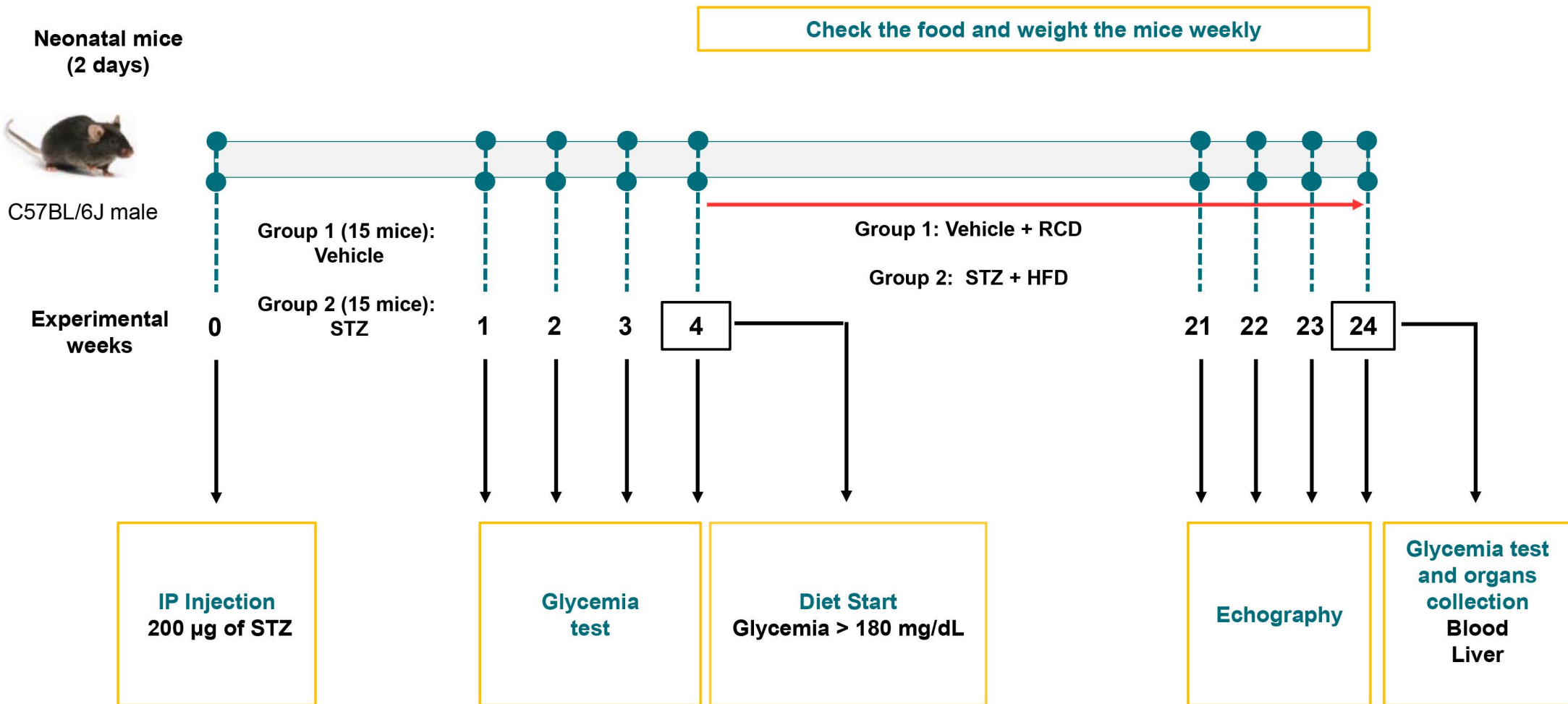
Figures Legends

Figure 1. Scheme of the procedure to induce HCC by streptozotocin and HFD. Neonatal (2 days old) mice are treated with vehicle (citrate buffer, group 1) or streptozotocin (STZ, group 2). After 1 week, glucose in plasma is analyzed from all groups. On week 4, glycemia is tested to discard non-diabetic mice (>180 mg/dL) from the group 2 and the animals are fed with high-fat diet (HFD). As a control, group 1 is fed with regular chow diet (RCD). All mice are fed for 20 weeks. From weeks 21 to 24, tumor growth is monitored by weekly echography. All animals are sacrificed 24 weeks after the beginning of the experiment.

Figure 2. Representative pictures of the liver with HCC derived by Diabetes/NAFLD. A) The development of the hepatic tumor is analyzed by echography in mice (treated with vehicle plus RCD) and in mice from the experimental group (treated with STZ plus HFD) before sacrifice. B) Representative photographs of the livers from the control and experimental groups are shown. The tumor area is labeled by a red circle.

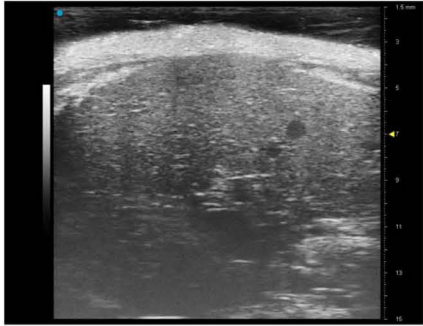
Type 2 Diabetes Mellitus Progression

NAFLD/HCC Progression

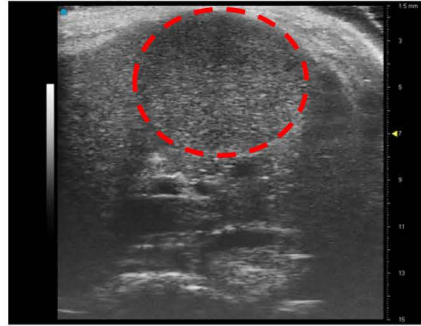


A

Vehicle + RCD

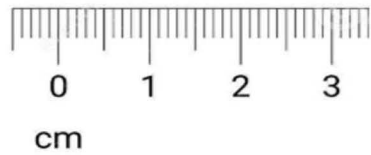


STZ + HFD



B

Vehicle + RCD



STZ + HFD

