Protocol

Optimized protocol for an inducible rat model of liver tumor with chronic hepatocellular injury, inflammation, fibrosis, and cirrhosis

Animal models of liver cancer are instrumental in the study of hepatocarcinogenesis and development of novel therapeutic approaches. Here, we describe steps to establish liver cancer in a rat model, via chronic administration of diethylnitrosamine. This causes liver tumors with a sequential progression of hepatitis, cirrhosis, and tumor formation, which closely mimics the development of human liver cancer. This protocol was optimized to significantly increase the incidence of liver tumor formation and reduce the duration of the procedure.

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HIGHLIGHTS

Detailed protocol for a carcinogen-induced rat hepatocellular carcinoma model

Undergoes a sequential progression of hepatitis, cirrhosis, and tumor formation

Optimized to significantly increase the incidence of liver tumor formation

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Optimized protocol for an inducible rat model of liver tumor with chronic hepatocellular injury, inflammation, fibrosis, and cirrhosis

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SUMMARY

Animal models of liver cancer are instrumental in the study of hepatocarcinogenesis and development of novel therapeutic approaches. Here, we describe steps to establish liver cancer in a rat model, via chronic administration of diethylnitrosamine. This causes liver tumors with a sequential progression of hepatitis, cirrhosis, and tumor formation, which closely mimics the development of human liver cancer. This protocol was optimized to significantly increase the incidence of liver tumor formation and reduce the duration of the procedure.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2020).

BEFORE YOU BEGIN

@ Timing: 1–2 h

1. Plan the experiment carefully and ensure sufficient group size (the number of rats per experimental and control group).
2. Weigh the rats and distribute them equally into different groups: the control group, hepatitis group, cirrhosis group, and carcinoma group.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins |        |            |
| Diethyl nitrosamine (DEN) | Sigma-Aldrich | Cat#N0258 CAS#55-18-5 |
| Formalin | Sinopharm Chemical Reagent | Cat#10010018 |
| PBS | Sangon | Cat#A610100-0001 |
| Sterile isotonic saline | Sinopharm Chemical Reagent | Cat#SY17103 |
| Hematoxylin and eosin staining kit | Beyotime Biotechnology | Cat#C0105S |

(Continued on next page)
**MATERIALS AND EQUIPMENT**

Sex- and age-matched 5- to 6-week-old rats of body weight 120–150 g and the same strain, such as Sprague-Dawley or Wistar strains (Troubleshooting 1). Here we recommend male rats in young age which are more susceptible to DEN.

**Note:** Similar to humans, HCCs develop faster and more frequently in males than in females (Newell et al., 2008; Caviglia and Schwabe, 2014).

**Note:** Usually, it is recommended to use the animals after they have reached their sexual maturity (10–12 weeks old). However, after sexual maturity, the immune system of rats is fully developed. Rats in advanced age are relatively resistant to developing HCC.

**Note:** A minimal of six animal per group is recommended (control, hepatitis, and cirrhosis). For the end point experiments (carcinoma), 12 animals is recommended.

**Note:** All experiments involving rats must conform with local and national animal care regulation.

**DEN working solution**

| Reagent                  | Final concentration | Amount  |
|--------------------------|---------------------|---------|
| DEN (0.95 g/mL)          | 70 mg/mL            | 1.05 mL |
| Sterile isotonic saline  | N/A                 | 13.2 mL |
| Total                    | N/A                 | 14.25 mL|

**Note:** This step should be done in sterile conditions.

**Note:** Gently mix the solution. Store DEN solution at 4°C for 2 weeks.

**Note:** Critical reagent.

⚠ **CRITICAL:** DEN is a very toxic and potent hepatocarcinogenic. Preparation of DEN solution should be performed in a chemical fume hood under appropriate protection, such as wearing face shields, gloves, and eyes shields. Do not inhale or expose it to the skin and eyes. Store the DEN solution at 4°C (cold room, refrigerator) in closed glass containers.
Note: Gently mix the solution. Store pentobarbital sodium solution at 4°C for at least 6 months.

△ CRITICAL: Pentobarbital Sodium is a short-acting barbituric acid derivative with central nervous system (CNS) depressant property, and used as an anesthetic and sedative in experimental animals. Pentobarbital sodium appears as crystalline granules or white powder. Preparation of pentobarbital sodium solution should be performed in a chemical fume hood under appropriate protection, such as wearing face shields, gloves, and eye shields. Do not inhale or expose it to the skin and eyes. Store the solution at 4°C (cold room, refrigerator) in closed glass containers.

### STEP-BY-STEP METHOD DETAILS

Most models of experimental liver carcinogenesis use mice. However, they commonly lack the typical features of chronic human liver disease that promote HCC development such as chronic injury, fibrosis, and cirrhosis. In humans, HCC develops in a chronically injured liver in 80% of the cases. In addition, mice develop HCCs with a low incidence when treated with DEN (Heindryckx et al., 2009).

Chronic administration of DEN in rats causes liver tumors with a sequential progression of hepatitis, cirrhosis, and tumor formation. Comparing to the 34% of induction rate for mice, recent studies demonstrated that Sprague-Dawley (SD) rats treated with 10 weekly doses of DEN (70 mg/kg body weight, intraperitoneally) develop HCC with up to 80% (Caviglia and Schwabe, 2014). This DEN-induced rat HCC model closely mimics the development of human liver cancer, which typically involve sequential stimuli for initiation and progression (Magee and Barnes, 1956). This protocol is aimed to generate a genotoxic hepatocarcinogenesis model in rats (Figure 1).

#### Induction of chronic liver injury and inflammation in rats

© Timing: 6 weeks

Reagent setup, labeling, weight measurements, and injections of DEN solution usually do not take more than 2 h. Inflammatory infiltration can be detected at the fourth to fifth week after the first DEN administration.

1. Put three rats in one cage and label the rats with ear markings or other similar methods.
2. Keep rats on a basal diet for one week.
3. Weigh the rats and calculate the amount of DEN needed.
4. Prepare as many aliquots of DEN working solution as necessary (70 mg per kg body weight, i.e., 180 μL working solution per 180 g rat body weight).
5. Tightly hold the rat in a vertical position. Place the body of the rat straight and catch the head with thumb and index finger. While holding the rat with one hand, apply with the other hand. Inject intraperitoneally DEN working solution for the experimental group (including hepatitis, cirrhosis, and carcinoma groups) or sterile isotonic saline for the control group using a 1 mL insulin syringe in a laminar airflow hood.

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| Reagent          | Final concentration | Amount |
|------------------|---------------------|--------|
| Pentobarbital sodium | 15 g/L              | 1.5 g  |
| ddH₂O            | N/A                 | 100 mL |
| Total            | N/A                 | 100 mL |
DEN may cause cancer and heritable genetic damage. Wear suitable protective clothing, gloves and face/eye protection. All handling, particularly the i.p. injections, should be performed in a laminar airflow hood.

Note: Avoid bubbles in the syringe.

CRITICAL: Ensure that the position of the rat is secured before inserting the insulin syringe. It is essential to ensure that the appropriate dose of DEN solution are injected completely into abdominal cavity (see Troubleshooting 2).

6. Put the rat back to the original cage with the regular diets, and closely monitor the health condition of the rat every day or every other day.
7. Repeat steps 3–6 at day 15 of the experiment.
8. Repeat steps 3–6 at day 22 of the experiment.
9. Repeat steps 3–6 at day 29 of the experiment.
10. Repeat steps 3–6 at day 36 of the experiment.

CRITICAL: DEN is very reliable in inducing liver carcinoma with high reproducibility and low mortality. However, sometimes the rats that are too young or too low in body weight or highly susceptible to DEN may die in the first few weeks after DEN treatment. Determination of weight every day or every other day is important to get an idea of hepatitis severity. If the weight is <90% of the initial weight, this is an indication that rat should be omitted from further experimentation, or dose of DEN may be decreased in subsequent experiments (see Troubleshooting 3).

11. At day 36 of the experiment, rats from hepatitis group were sacrificed with 1.5% (wt/vol) pentobarbital sodium (30 mg/kg) by intraperitoneal injection.
12. Place rats on the dissection tray and secure limbs using strings (Figure 2).
13. Using forceps and scissors, open the abdominal cavity and dissect out the liver carefully.
14. Remove the entire liver using an anatomical forceps and a scalpel or scissors, and place the liver in a 10 cm Petri dish filled with PBS.

Note: Avoid to bruise the liver, holding the ligaments of the liver with the forceps.

15. Rinse the liver with PBS twice to remove the remaining blood.
16. The entire liver of each rat was observed grossly and weighed (Figure 3).
17. The livers from each rat were collected and divided into pieces. Some samples of frequently used techniques are described below: Preparation for subsequent molecular biological investigations (option a) and histopathological analysis (option b).
   a. Preparation for molecular biological investigations (e.g., for DNA sequence analysis, RNA sequence analysis, and western blot).
      i. Carefully and quickly snap-frozen the liver tissue directly in liquid nitrogen.
      ii. Proceed directly with DNA, RNA or protein purification using standard procedures or stored at \(-80^\circ\text{C}\).
   b. Histopathological analysis
      i. The remaining pieces were preserved in 10% phosphate-buffered formalin.
      ii. Embed in paraffin and prepare 5 \(\mu\text{m}\) paraffin cross-sections.
      iii. Mount the slices on glass slides.
      iv. Stain sections with hematoxylin and eosin (H&E) using standard procedures.
      v. Assess necroinflammation, fibrosis, cirrhosis, and tumor formation by an experienced pathologist.

Pause point: Extracted genomic DNA, RNA or protein can be stored long-term at \(-80^\circ\text{C}\) for later analysis.

b. Histopathological analysis
   i. The remaining pieces were preserved in 10% phosphate-buffered formalin.
   ii. Embed in paraffin and prepare 5 \(\mu\text{m}\) paraffin cross-sections.
   iii. Mount the slices on glass slides.
   iv. Stain sections with hematoxylin and eosin (H&E) using standard procedures.
   v. Assess necroinflammation, fibrosis, cirrhosis, and tumor formation by an experienced pathologist.

Induction of fibrosis and cirrhosis in rats

Timing: 9 weeks

18. Repeat steps 3–6 at day 43 of the experiment.
19. Repeat steps 3–6 at day 50 of the experiment.
20. Repeat steps 3–6 at day 57 of the experiment.
21. Repeat steps 3–6 at day 64 of the experiment.
22. Repeat steps 3–6 at day 71 of the experiment.
23. Eight weeks after the first injection, liver fibrosis start to occur. At appropriate time points, the rats from selected group were sacrificed with 1.5% (wt/vol) pentobarbital sodium (30 mg/kg) by intraperitoneal injection. (see Troubleshooting 2).

24. Repeats steps 12–17 (Figure 3).

CRITICAL: When you establish the HCC model for the first time, include an additional subgroup of rats in order to confirm the sequential progression state of the liver. Sacrifice rats with pentobarbital sodium, collect the livers, and fix them in neutral formalin. After embedding in paraffin, slice the paraffin block, and stain with H&E to assess liver hepatitis, fibrosis, and cirrhosis state. Select the appropriate time points according to the assessment results and the purpose of the experiment (see Troubleshooting 4).

25. In the cirrhosis models, analysis at the 14th week after the first injection may be appropriate in most cases. At this time point, nodular cirrhosis could be seen macroscopically. (see Troubleshooting 2)

Spontaneous tumor progression and preparation for tumor analysis

© Timing: 10 weeks

26. Carefully monitor the animals, determine the weight of the rats and watch for signs of distress.

Note: At this time point, experimental rats should have gain of weight after DEN administration. However, if the experimental rat loss of weight and display signs of overt distress, it should be killed in time for further analysis.
27. Twenty four weeks after the first injection, the rats were sacrificed with 1.5% (wt/vol) pentobarbital sodium (30 mg/kg) by intraperitoneal injection.

△ CRITICAL: At this time point, the experimental rats were obviously thin and small, compares with the control group. However, they were still large (Figure 4), and showed restlessness, irritability, aggressiveness. Carefully hold the rats when doing experiments to avoid being bitten by rats.

28. Place rats on the dissection tray and secure limbs using strings.
29. Using forceps and scissors, open the abdominal cavity and dissect out the liver carefully.
30. Remove the entire liver using an anatomical forceps and a scalpel or scissors, and place the liver in a 10 cm Petri dish filled with PBS.
31. Rinse the liver with PBS twice to remove the remaining blood.
32. The entire liver of each rat was observed grossly and weighed (Figure 3).
33. Harvest tumors and adjacent normal control tissue. (see Troubleshooting 1).
34. Some tissues were snap-frozen directly in liquid nitrogen and stored at −80°C. The remaining pieces were preserved in 10% phosphate-buffered formalin for histopathologic examinations.
35. Proceed directly with DNA, RNA or protein purification using standard procedures or stored at −80°C.

Pause point: Extracted genomic DNA, RNA or protein can be stored long-term at −80°C for later analysis.
CRITICAL: Rats receiving chronic administration of DEN develop HCC by 36 weeks with an incidence of 80%. If less than 60% of the animals develop liver carcinoma, try to extend the experiment time, even until by the time of death (see Troubleshooting 5).

EXPECTED OUTCOMES

The rat model of DEN-induced hepatocarcinogenesis has helped in our understanding of molecular events in HCC. Importantly, this model closely mirrors human liver cancer development, which undergoes a sequential progression of toxic hepatitis, fibrosis or cirrhosis, and ultimately HCC. So it allow us to further identify key cell populations involved in this process and define genetic drivers of human HCC. Here, we used a well-established protocol to generate a genotoxic hepatocarcinogenesis (Solt et al., 1977; Solt et al., 1983; Scherer and Emmelot, 1976).

In this experimental model, 36 male Sprague-Dawley (SD) rats were administered 10 once-weekly doses of DEN for 10 weeks. Twelve rats were sacrificed at 5th weeks, 13th/14th weeks, and 23rd/24th weeks, respectively.

At the 5th week after the first DEN administration, inflammatory infiltration occurred in 9 of 12 rats (Figure 5). Pathological histology in livers from this group showed hepatocyte swelling, expansion of some portal areas, and inflammatory cell infiltrating in portal duct areas (Figure 6). At the 14th week, liver cirrhosis occurred in 10 of 12 rats, and one of 12 rats developed HCC (Figure 5). Results of pathological sections showed fibrous tissue hyperplasia, fibrous bands, and significant portal-to-portal and portal-to-central bridging with pseudolobular formation in livers from this group (Figure 6). At the 24th week, 7 of 12 rats developed multiple, macroscopically identifiable tumors, and among which 6 developed HCC (Figure 5). Tumor nodule from livers showed tumor cells arranged irregularly, small cell change with high cell density and cytological atypia (Figure 6). Furthermore, livers contained not only HCC but also adenocarcinoma histologically at the 24th week.

The pathologic changes to the liver in rats treated with DEN resembled those associated with human hepatocarcinogenesis. A comparison of carcinogen-induced rat mutations by using whole-exome sequencing with TCGA liver HCC revealed substantial overlap in the enrichment of signaling pathway genes harboring consequential mutations. The somatic mutational landscape of DEN-induced rat HCC reflects the tumor architecture in human HCC (Chen et al., 2020).

Figure 5. Breakdown of different histological findings in each treatment group

△ CRITICAL: Rats receiving chronic administration of DEN develop HCC by 36 weeks with an incidence of 80%. If less than 60% of the animals develop liver carcinoma, try to extend the experiment time, even until by the time of death (see Troubleshooting 5).
Thus, the evaluation of HCC by advanced molecular biological methods, such as the second generation, third generation sequencing technology, will allow a better and more sensitive characterization of tumor development. This model may also be useful for preclinical testing of cancer therapeutics.
LIMITATIONS
It is essential to ensure that the appropriate dose of DEN solution is completely injected into abdominal cavity. Ensure the position of the rat before inserting the insulin syringe. When inserting the syringe into the abdominal cavity, the rats will struggle. Make sure that the needle remains at the right position in the abdominal cavity while injecting the DEN solution.

In addition, to get reproducible results, determining the body weight of the rats and watching for signs of distress are frequently important in estimating the disease severity. In most cases, experimental rats should have increased the weight slowly after the first injection. However, if the weight is <90% of the initial weight, rats should be omitted from further experimentation, or dose of DEN for this rat may be decreased in subsequent experiments.

Generally, it is difficult to distinguish fibrosis and cirrhosis strictly. They always occur simultaneously.

TROUBLESHOOTING
Problem 1
No or very few tumors.

Potential solution
This is one of the most common reasons for failure of the protocol. Try to switch to a rat strain with higher susceptibility. F344 rats are more susceptible to DEN-induced carcinogenesis than Wistar or Brown Norway rats.

Problem 2
High variability within experimental groups

Potential solution
It is essential to ensure that the appropriate dose of DEN solution is completely injected into the abdominal cavity. Ensure precise injection technique. Tightly hold the rat in a vertical position, and place the body of the rat straight. While holding the rat with one hand, apply with the other hand. Ensuring that the needle remains at the right position in the abdominal cavity while injecting the DEN solution. This procedure should be performed by well-trained personnel.

Problem 3
Rats become weak or show signs of compromised health status.

Potential solution
Carefully monitor the animals, performing daily determination of weight and watching for signs of distress. If the weight is <90% of the initial weight, rat should be omitted from further experimentation, or dose of DEN for this rat may be decreased in subsequent experiments.

Problem 4
Hard to confirm the progression state of the liver based on the number of weeks after DEN treatment.

Potential solution
Usually, inflammatory infiltration occurred at the 5th week after the first DEN administration. Liver fibrosis and cirrhosis occurred at the 14th week after DEN administration. However, the time of when the rats display hepatitis, fibrosis, and cirrhosis depend on the sensitivity to DEN treatment. For example, rats, which are too low in body weight or highly susceptible to DEN, may develop fibrosis within 5 weeks. So when you establish the HCC model for the first time, include an additional subgroup of rats in order to confirm the sequential progression state of the liver. Three weeks after the first injection, sacrifice one or two rats every week to assess hepatitis, fibrosis, and cirrhosis state.
Problem 5
Low incidence of tumor formation.

Potential solution
If less than 60% of the animals develop liver carcinoma, try to extend the experiment time. Try to sacrifice the rats at 32 weeks after the first injection. The incidence of tumor formation may optionally be noninvasively monitored by in vivo imaging of tumor using an IVIS Spectrum CT system.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xianghuo He (xhhe@fudan.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS
Z.C. and S.L. helped in protocol optimization and wrote the manuscript. H.L. and X.H. developed the protocol and revised the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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