Journal of Basic and Clinical Physiology and Pharmacology

Home

Author

Review

Guest Editor Center

Author Dashboard

2 Manuscripts with Decisions

Start New Submission

Legacy Instructions

5 Most Recent E-mails

English Language Editing Service

Manuscripts with Decisions

ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
	ME: Marra, Alberto AME: Marra, Alberto • Accept (08-Mar-2021) *Archiving completed on 16-Oct-2021* view decision letter Contact Journal	JBCPP.2020.0475.R1	N-nitrosodiethylamine induces inflammation of liver in mice *Files Archived*	17-Feb-2021	08-Mar-2021
a revision has been submitted (JBCPP.2020.0475.R1)	ME: Marra, Alberto ME: Appelt, Katharina AME: Not Assigned • Revise with Major Modifications (31-Dec-2020)	JBCPP.2020.0475	Animal model of liver disease in mice induced with n-nitrosodiethylamine *Files Archived*	29-Nov-2020	31-Dec-2020

ACTION	STATUS	ID	TITLE	SUBMITTED	DECISIONED
	• a revision has been submitted *Archiving completed on 16-Oct-2021* view decision letter Contact Journal				

andang miatmoko <andang-m@ff.unair.ac.id>

JBCPP.2020.0475 - DecisionRevise with Major Modifications

1 message

Journal of Basic and Clinical Physiology and Pharmacology <onbehalfof@manuscriptcentral.com>
Reply-To: jbcpp.editorial@degruyter.com
To: andang-m@ff.unair.ac.id
Cc: scientificicph@ff.unair.ac.id

Thu, Dec 31, 2020 at 2:30 PM

31-Dec-2020

Dear Dr. Miatmoko:

Thank you again for submitting your manuscript ID JBCPP.2020.0475 entitled "Animal model of liver disease in mice induced with n-nitrosodiethylamine" to Journal of Basic and Clinical Physiology and Pharmacology (JBCPP). Your manuscript has been reviewed and requires major modifications prior to acceptance. The comments of the reviewer(s) are included at the bottom of this letter.

I invite you to respond to the reviewer(s)' comments and revise your manuscript.

To revise your manuscript, log into https://mc.manuscriptcentral.com/jbcpp and enter your Author Center, where you will find your manuscript title listed under "Manuscripts Awaiting Revision". Under "Actions", click on "Create a Revision". Your manuscript number has been appended to denote a revision.

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2. and/or a rebuttal against each point that is being raised

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Once again, thank you for submitting your manuscript to JBCPP. I look forward to receiving your revision.

Kind regards
Dr. Suciati Suciati
Guest Editor, Journal of Basic and Clinical Physiology and Pharmacology

Reviewer(s)' Comments to Author:

Reviewer: 1

Comments to the Author
The research describes the modeling for NDEA induced liver injury. The method and result have been described sufficiently. However, the model has been described elsewhere, e.g. doi: 10.1515/intox-2015-0001. It is important to make a good note in the manuscript about the novel finding in the study. The authors must focus on the liver disease if it is what they aim for. Following are the notes:
1. What is the new finding filling the gap in the field. Reports are showing the success of the model in other studies.
2. It is important to redefine the focus. In the title, it is written as liver disease, which varies in types. The background of the study implies the aim is liver cancer disease. The result showed many organ profiles. The histology data showed liver and spleen. This scrambled data and inconsistency should be adjusted to prevent confusion.
3. In the table 1 caption, the author should put the number of the sample (n) as the number of animal in 1 group, not all group. Further, the author should add the information of the value presentation of the result, whether it is mean ± SD or mean ± SEM, or else. For the normal group results, the result should be added by SEM or SD value compared to the NDEA group.
4. It is also important to include the Error/ Deviation bar for the Normal group line in figure 1 and the statistical mark for the comparison to the normal group. It is assumed that the normal group line was made by the mean of some samples.
5. The author explains about cancer-related cachexia represented by the weight loss data. This is irrelevant since there is no evidence of cancer formation in the present finding.
6. It is not stated elsewhere whether the method is purposively made as short term induction. It should be defined whether the term of treatment should be in purpose to describe the targeted features in the model.
7. Decrease of mice organ is possible because NDEA is also able to induce tumors in various organs such as the lungs, liver, esophagus, kidneys, stomach, intestines, and nervous system. This explanation is somehow made confusing. Again, there is no evidence of cancer occurring in the model. The author should make a logical explanation regarding the change in organ weight.
8. There is no relevance in featuring non-liver organ's weight. There is no correlative explanation of why it is done.

Reviewer: 2

Comments to the Author
Cohesion between sentences not good enough. Please proofread this paper.
Please look at figure 2, "The visual observation of normal liver (B) and the liver after NDEA induction (B) of mice." Is it true?
NDEA is well known to induce hepatotoxicity in the preclinic study, is it any different valuable information to strengthen this study?
There is a very weak to relate between weight loss and hepatocarcinogens.
There is very lack of evidence in this result study that NDEA induced hepatocarcinogens, pathology anatomy in surrounding tissue assessment must be provided to support this statement, not only morphology and he evaluation.
Give a logical explanation about the length of NDEA intervention that can produce a carcinogenic cell.
How many replications in this study?

andang miatmoko <andang-m@ff.unair.ac.id>

JBCPP.2020.0475.R1 - DecisionAccept

1 message

Journal of Basic and Clinical Physiology and Pharmacology <onbehalfof@manuscriptcentral.com>
Reply-To: jbcpp.editorial@degruyter.com
To: andang-m@ff.unair.ac.id
Cc: scientificicph@ff.unair.ac.id

Mon, Mar 8, 2021 at 7:10 PM

08-Mar-2021

Dear Dr. Miatmoko:

I would like to thank you for submitting your manuscript entitled "N-nitrosodiethylamine induces inflammation of liver in mice" to Journal of Basic and Clinical Physiology and Pharmacology (JBCPP). Your manuscript has been reviewed, and it is a pleasure to accept it for publication in JBCPP.

We require publication charges to cover our editorial and production expenses.The publication charges are 3.500.000 IDR or 250 USD or 1025 MYR for the accepted article. You are required to process with publication charges upon acceptance of your article (no later than 5 days after acceptance letter). Please upload proof of payment through the following link: http://bit.ly/39bcHl2
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The JBCPP production office will contact you for proofreading in the near future. Your article will be published ahead of print as soon as possible, and assigned to an online issue at a later time.

Thank you for your fine contribution. On behalf of the Editors of Journal of Basic and Clinical Physiology and Pharmacology we look forward to your continued contributions to the Journal.

Kind regards
Dr. Suciati Suciati
Guest Editor, Journal of Basic and Clinical Physiology and Pharmacology

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DE GRUYTER Journal of Basic and Clinical Physiology and Pharmacology

N-nitrosodiethylamine induces inflammation of liver in mice

Journal:	*Journal of Basic and Clinical Physiology and Pharmacology*
Manuscript ID	JBCPP.2020.0475.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Cahyani, Devy Maulidya; Universitas Airlangga Fakultas Farmasi, Pharmaceutics Miatmoko, Andang ; Universitas Airlangga Fakultas Farmasi, Pharmaceutics; Faculty of Pharmacy, Airlangga University Hariawan, Berlian Sarasitha; Universitas Airlangga Fakultas Farmasi, Pharmaceutics Purwantari, Kusuma Eko; Universitas Airlangga Fakultas Kedokteran, Department of Anatomy and Histology Sari, Retno; Universitas Airlangga Fakultas Farmasi,
Section/Category:	• Oxidative Stress
Keywords:	liver, mice, n-nitrosodiethylamine, inflammation
Abstract:	Objectives: For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction. Methods: The BALB-c mice were induced with NDEA 25mg/kg of body weight once a week for five weeks intraperitonially and it was then evaluated for the body weight during study periods. The mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances and histopathology evaluations. Results: The results showed a significant decrease of body weight of mice after 5 times induction of 25 mg NDEA/kgBW per week intraperitonially. Different morphological appearances and weight of mice organs specifically for liver and spleen had also been observed. The histopathology examination showed that there were hepatic lipidosis and steatohepatitis observed in liver and spleen, respectively that might indicate the hepatocellular injury. Conclusions: It can be concluded that inducing mice with NDEA intraperitonially resulted in fatty liver disease leading to progress of cancer disease.

Dear Editor,
Many thanks for the email. We really appreciate all comments to improve our manuscript. Below are the answers addressed for the reviewer's comment.

Reviewer: 1
Comments to the Author

The research describes the modeling for NDEA induced liver injury. The method and result have been described sufficiently. However, the model has been described elsewhere, e.g. doi: 10.1515/intox-2015-0001. It is important to make a good note in the manuscript about the novel finding in the study. The authors must focus on the liver disease if it is what they aim for. Following are the notes:

1. What is the new finding filling the gap in the field. Reports are showing the success of the model in other studies.
Answer:
In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer development. The reports on the use of NDEA for making liver inflammation itself are limited. For the future study, we would like to have some models for the treatment, including preventive and curative actions. The cancer models itself has been reported by induction of NDEA at a dose of 25 mg/Kg BW for 8 weeks, while the preventive mode is still a limited study.

We have revised the title into: "N-Nitrosodiethylamine induces liver inflammation in mice"

We have revised and added some sentences I page 1 line 6-10 as the following:
"For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction"

2. It is important to redefine the focus. In the title, it is written as liver disease, which varies in types. The background of the study implies the aim is liver cancer disease. The result showed many organ profiles. The histology data showed liver and spleen. This scrambled data and inconsistency should be adjusted to prevent confusion.
Answer:
In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer progression. So, we focused on liver and spleen as the target organs reflecting the inflammation model induced by NDEA intraperitoneal injection. However, we would like to show other organs to see whether there are any physical changes, which non-different morphologies were visually observed after the observation for lungs, heart, and kidney.

We have revised the title into: "N-Nitrosodiethylamine induces liver inflammation in mice"

We have revised and added some sentences I page 1 line 6-10 as the following:

"For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction"

We have revised and added some sentences into the background in line 27-50 as the following:
"The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumour initiation, growth, development, and metastasis [6]. Inflamation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte / macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].
In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic immune system, allowing cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].

Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a "secretome" that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].

The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC."

We have also added sentences in line 52-54 as the following:
"Preventive care could be highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice is often used for animal model, especially for cancer research [11]."

We have also added sentences in line 68-69 as the following:

"NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy"

3. In the table 1 caption, the author should put the number of the sample (n) as the number of animal in 1 group, not all group. Further, the author should add the information of the value presentation of the result, whether it is mean ± SD or mean ± SEM, or else. For the normal group results, the result should be added by SEM or SD value compared to the NDEA group.

Answer:

Many thanks for the comments. We have added the sampel number in each figure or table legends, as the following:

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). ***P*<0.05..

Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice.

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

We have also added the "Organ weights (mean ± SD)" in the column title of Table 1.

In line 91, we have revised the sentence as the following:
"The results were presented as the mean ± SD"

4. It is also important to include the Error/ Deviation bar for the Normal group line in figure 1 and the statistical mark for the comparison to the normal group. It is assumed that the normal group line was made by the mean of some samples.

Answer:

We have revised the figure 1 and Table 1 by adding standard deviation of measurement, especially for the control or normal group.

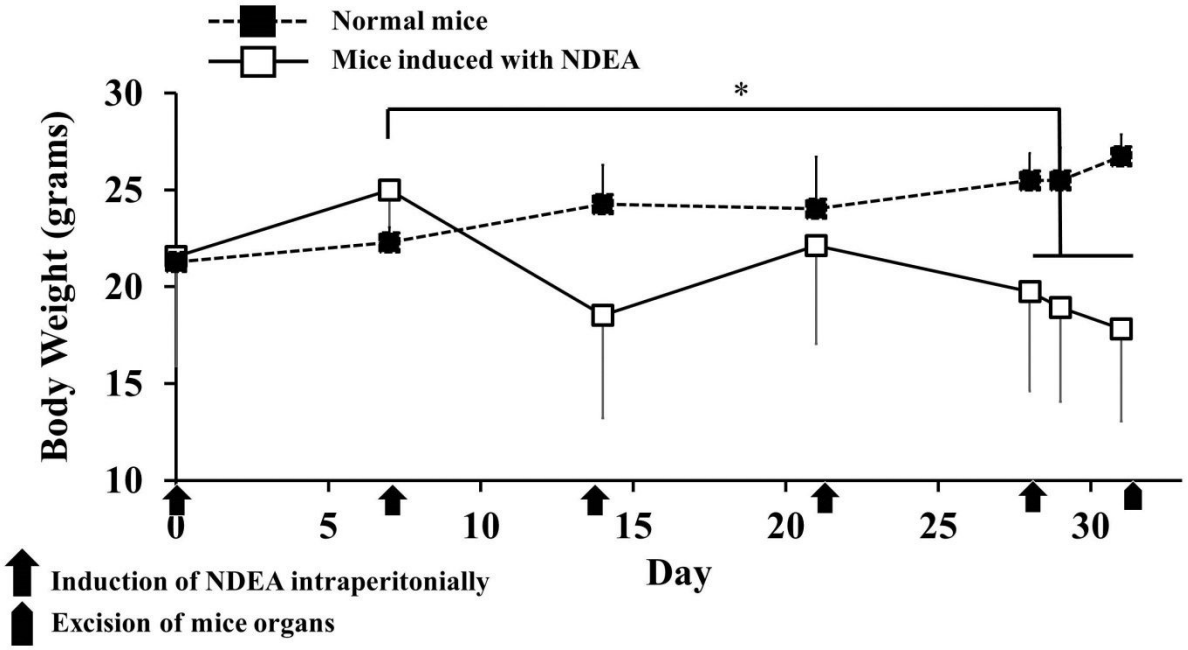

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). $^{**}P<0.05$.

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

Organ	Organ weights (mean ± SD)	
	Normal	After NDEA Induction
Heart	0.11 ± 0.01 g	0.08 ± 0.03 g
Lungs	0.20 ± 0.04 g	0.32 ± 0.05 g
Liver	1.86 ± 0.13 g	0.97 ± 0.27 g
Spleen	0.23 ± 0.12 g	0.20 ± 0.12 g
Kidney	0.40 ± 0.05 g	0.25 ± 0.06 g

5. The author explains about cancer-related cachexia represented by the weight loss data. This is irrelevant since there is no evidence of cancer formation in the present finding.

Answer:

According to the previous study, induction of NDEA resulted in lesser food intake of mice than the normal group causing the weight losses. We have revised and added discussion about this in line 143-147 as the following:

"It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease

as target for preventive cure of naticancer agents. NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [23]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [24]. However, in this study, there was no evaluation of food consumed by the mice during the experiments..”

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, “The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis,” *Heliyon*, vol. 5, no. November 2018, p. e01769, 2019.

[23] N. S. Thomas, K. George, S. Arivalagan, V. Mani, A. I. Siddique, and N. Namasivayam, “The in vivo antineoplastic and therapeutic efficacy of troxerutin on rat preneoplastic liver: biochemical, histological and cellular aspects,” *Eur. J. Nutr.*, vol. 56, no. 7, pp. 2353–2366, 2016.

[24] V. Rajesh and P. Perumal, “Chemopreventive and antioxidant activity by Smilax zeylanica leaf extract against N-nitrosodiethylamine induced hepatocarcinogenesis in wistar albino rats,” *Orient. Pharm. Exp. Med.*, vol. 14, no. 2, pp. 111–126, 2014.

6. It is not stated elsewhere whether the method is purposively made as short term induction. It should be defined whether the term of treatment should be in purpose to describe the targeted features in the model.

Answer:

In this study, the short term induction refers to shorter periods of NDEA induction, which was 5 weeks, than the previous study that stated 8 weeks induction of NDEA produced liver cancer. According to this comment, we have revised the definition of short term by changing “short term: with “after 5 weeks”, as the following:

Page 1 Line 9: “induced with hepatocarcinogenic NDEA for 5 weeks induction”

Line 69-70: “Thus, this study aimed to evaluate the liver disease model observed in mice induced with hepatocarcinogenic NDEA for 5 weeks intraperitonal injection.”

Line 145-146: “NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1).”

Line 162-163: “However, in this study, instead of malignancies, hepatic lipidosis and steatohepatitis were observed in mice liver and spleen after 5 weeks induction of NDEA”

Line 170: “stage of liver disease after 5 weeks induction of NDEA.”

Line 172: “Induction of NDEA in mice for 5 weeks”

7. Decrease of mice organ is possible because NDEA is also able to induce tumors in various organs such as the lungs, liver, esophagus, kidneys, stomach, intestines, and nervous system. This explanation is somehow made confusing. Again, there is no evidence of cancer occurring in the model. The author should make a logical explanation regarding the change in organ weight.

Answer:

Thank you for the comment. In this study, there were no significant different of organ weights of heart, lungs, kidney, and spleen between control and NDEA induction group, however, after NDEA induction, the liver weight was significantly decreased.

We have revised by deleting those statements in the discussion section, and revised the discussion in line 152-156 as the following:

”NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [25]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [26]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group.”

[25] G. Mittal, A. P. S. Brar, and G. Soni, “Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: Biochemical and histopathological effects,” *Pharmacol. Reports*, vol. 58, no. 3, pp. 413–419, 2006.

[26] R. C. Cattley and J. M. Cullen, *Liver and Gall Bladder*. 2013. Chapter 45: *Liver and Gall Bladder*. In W.M. Haschek, C.G.Rousseaux, M.A. Wallig, B.Bolon, R. Ochoa & B.M. Wahler (Eds). Haschek & Rouseaux's Handbook of Toxicology Pathology (3rd). Boston: Academic Press

8. There is no relevance in featuring non-liver organ’s weight. There is no correlative explanation of why it is done.

Answer:

In this study, we excised all organs from the mice of the control and NDEA-induced groups to compare whether there were changes in physical appearances and organ weights, which may could be used for analyzing the effect of NDEA inducing inflammation to mice organs. It because of the ability of NDEA for inducing tumors in various organs such as the lungs, liver, esophagus, kidneys, stomach, intestines, and nervous system. However, there was significant different in liver visual appearance and liver weight as well as confirmed by histology evaluation. .

We have revised and added sentences in line 150-155 in the paragraph as the following:

“Based on the weight data for each organ shown in Table 1, it was known that the weight of liver organs in the treatment group decreased compared to control group. NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [25]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [26]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group”

[25] G. Mittal, A. P. S. Brar, and G. Soni, “Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: Biochemical and histopathological effects,” *Pharmacol. Reports*, vol. 58, no. 3, pp. 413–419, 2006.

[26] R. C. Cattley and J. M. Cullen, *Liver and Gall Bladder*. 2013. Chapter 45: *Liver and Gall Bladder*. In W.M. Haschek, C.G.Rousseaux, M.A. Wallig, B.Bolon, R. Ochoa & B.M. Wahler (Eds). Haschek & Rouseaux's Handbook of Toxicology Pathology (3rd). Boston: Academic Press

Reviewer: 2

Comments to the Author

1. Cohesion between sentences not good enough. Please proofread this paper.

Answer:

We have proofread the manuscript.

2. Please look at figure 2, "The visual observation of normal liver (B) and the liver after NDEA induction (B) of mice." Is it true?

Answer:

We have revised the figure legend as the following:

"Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice."

3. NDEA is well known to induce hepatotoxicity in the preclinic study, is it any different valuable information to strengthen this study?

In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer progression. So, we focused on liver and spleen as the target organs reflecting the inflammation model induced by NDEA intraperitoneal injection. However, we would like to show other organs to see whether there are any physical changes, which non-different morphologies were visually observed after the observation for lungs, heart, and kidney.

We have revised the title into: "N-Nitrosodiethylamine induces liver inflammation in mice"

We have revised and added some sentences I page 1 line 6-10 as the following:

"For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction"

We have revised and added some sentences into the background in line 27-50 as the following:

"The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumour initiation, growth, development, and metastasis [6]. Inflamation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte / macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].

In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic immune system, allowing cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the

resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].

Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a "secretome" that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].

The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC."

We have also added sentences in line 52-54 as the following:

"Preventive care could be highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice is often used for animal model, especially for cancer research [11]."

We have also added sentences in line 68-69 as the following:

"NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy"

4. There is a very weak to relate between weight loss and hepatocarcinogens.

Answer:

According to the previous study, induction of NDEA resulted in lesser food intake of mice than the normal group causing the weight losses. We have revised and added discussion about this in line 143-149 as the following:

"It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents. NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [23]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [24]. However, in this study, there was no evaluation of food consumed by the mice during the experiments."

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, "The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis," *Heliyon*, vol. 5, no. November 2018, p. e01769, 2019.

[23] N. S. Thomas, K. George, S. Arivalagan, V. Mani, A. I. Siddique, and N. Namasivayam, "The in vivo antineoplastic and therapeutic efficacy of troxerutin on rat preneoplastic liver: biochemical, histological and

cellular aspects," *Eur. J. Nutr.*, vol. 56, no. 7, pp. 2353–2366, 2016.
[24] V. Rajesh and P. Perumal, "Chemopreventive and antioxidant activity by Smilax zeylanica leaf extract against N-nitrosodiethylamine induced hepatocarcinogenesis in wistar albino rats," *Orient. Pharm. Exp. Med.*, vol. 14, no. 2, pp. 111–126, 2014.

5. There is very lack of evidence in this result study that NDEA induced hepatocarcinogens, pathology anatomy in surrounding tissue assessment must be provided to support this statement, not only morphology and the evaluation.

Answer:

In this study, we have evaluated the histopathology evaluations by haematoxylin-eosin staining for liver and spleen tissues. According to the results as shown in Figure 3, the normal liver and spleen have regular architecture and cellular integrity with no fibrosis. After induction of NDEA, there were no malignancies observed in liver on spleen tissues in mice; however, there were single large fat droplets, alongside nuclei dislocation to the cell periphery, seems to be macrovesicular steatosis. According to these results, there were lipidosis in liver and steatohepatitis observed for spleen tissue.

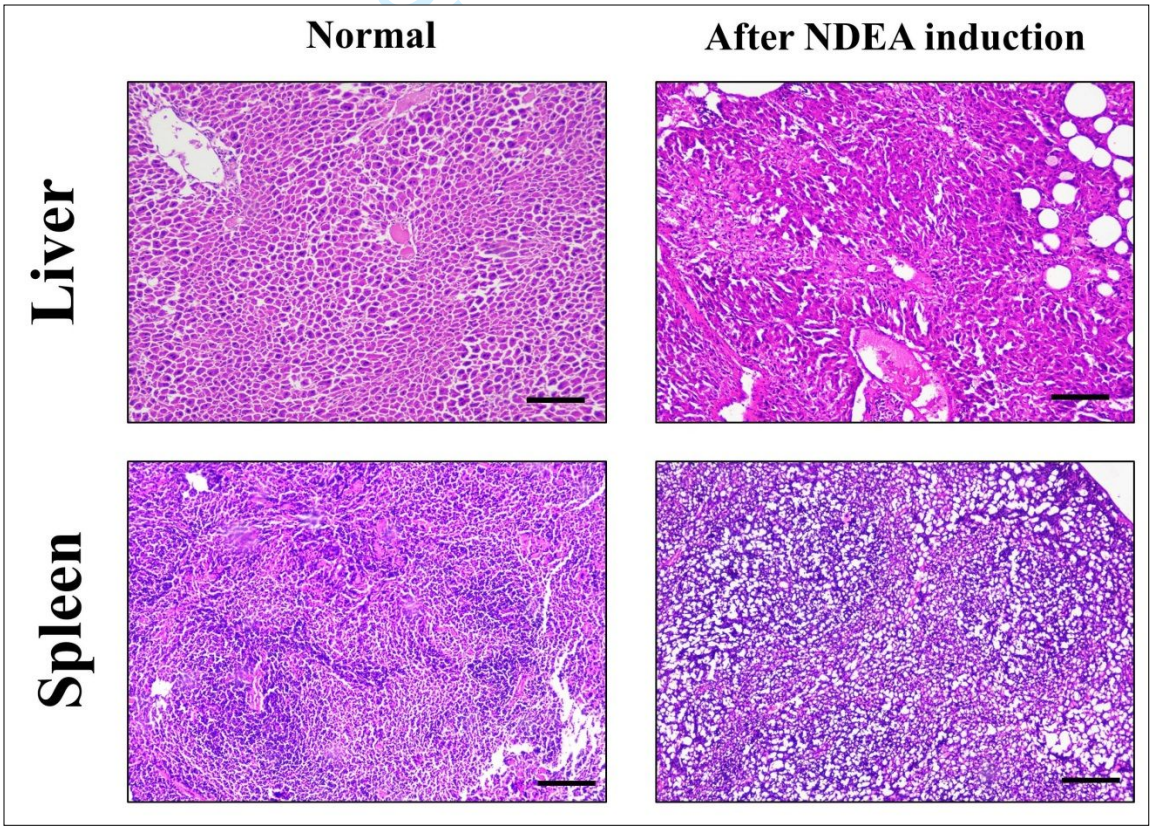

Figure 3: The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for 5 times. Scale bar= 100 µm.

6. Give a logical explanation about the length of NDEA intervention that can produce a carcinogenic cell. How many replications in this study?

Answer:

According to the previous study, the induction of NDEA for 8 weeks resulted in hepatocellular carcinoma indicated by enlarged hyperchromatic nucleus of hepatocytes in liver tissue and

scattered mitosis [22]. However, to produce an animal model for an early stage of cancer or preventive cure of cancer, in this study, NDEA was induced for 5 weeks and as indicated by the weight loss, the inflammation process has occurred.
We have added some discussion in line 143-145 as the following:
"It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents."
In this study, there were 7 mice in NDEA induction group and 3 mice for the control group.

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, "The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis," *Heliyon* vol. 5, no. May, 2019.

DE GRUYTER Journal Abbreviation. Year; short doi

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N-nitrosodiethylamine induces inflammation of liver in mice

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Abstract

Objectives: For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction.
Methods: The BALB-c mice were induced with NDEA 25mg/kg of body weight once a week for five weeks intraperitonially and it was then evaluated for the body weight during study periods. The mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances and histopathology evaluations.
Results: The results showed a significant decrease of body weight of mice after 5 times induction of 25 mg NDEA/kgBW per week intraperitonially. Different morphological appearances and weight of mice organs specifically for liver and spleen had also been observed. The histopathology examination showed that there were hepatic lipidosis and steatohepatitis observed in liver and spleen, respectively that might indicate the hepatocellular injury.
Conclusions: It can be concluded that inducing mice with NDEA intraperitonially resulted in fatty liver disease leading to progress of cancer disease.

Keywords: inflammation; liver; mice; n-nitrosodiethylamine

Introduction

Cancer is the world's leading health problem and the second leading cause of death in United States [1]. Cancer continues to increase worldwide, primary liver cancer is the leading cause of cancer with case about 841,000 new patients and causing 782,000 deaths in 2018 [2], [3]. There are two types of liver cancer, first *Hepatocellular carcinoma* (HCC) which causes 75% of all liver cancer cases and *Intrahepatic Cholangiocarcinoma* (ICC) which causes 12-15% of incidence [4]. HCC comes from hepatocytes, in which it is caused due to oxidative stress, inflammation, and is based on liver disease. On the other hand, ICC appears on c*holangiocyte* which is an intrahepatic bile duct [4], [5]. The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumour initiation, growth, development, and metastasis [6]. Inflamation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte / macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].

In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic immune system, allowing

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cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].

Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a "secretome" that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].

The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC.

Making animal models provides a great opportunity to study a disease as well as designing strategies for the treatment, whether it is preventive or curative actions [10]. Preventive care could highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice are often used for animal model, especially for cancer research [11]. This is because animals, especially rodents, have biological similarities both genetically and physiologically to humans. Therefore, the use of mice as experimental animal models is very suitable to identify the dangers caused by a xenobiotic or study the pathogenesis of a disease [12], [13].

The most common animal models of cancer are *xenograft* models [14]. However, the animals models using the *xenograft* model has a weakness, such as it can harm the immune system so it cannot represent cancer that occurs naturally in humans [11]. Another method of using mice as the inflammation disease model is the induction of hepatocarcinogen. Chemically, hepatocarcinogen can cause changes in the DNA structures and instability including N-Nitrosodiethylamine (NDEA), aflatoxine, carbon tetrachloride, dimethylnitrosamine, and thioacetamide. Inducing hepatocarcinogens using NDEA is a commonly used method for producing HCC animal model (11,12).

In liver, NDEA can induce progressive, proliferative, and mutagenic metabolism of tumors, so it can cause a wide variety of tumors in all animal models by intraperitoneal injection for about 8 weeks or more [17]. NDEA can produce pro-mutagenic products namely O^6-ethyl deoxy guanosine and O^4 and O^6-ethyl dioxy thymidine in the liver which are responsible for its carcinogenic effects [18]. NDEA, which is a chemical hepatocarcinogen, is also known to induce the Transforming Growth Factor Alpha (TGF-α) expression, which is closely involved in hepatocarcinogenesis and transformation in humans and animals [19]. NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy. Thus, this study aimed to evaluate the liver disease model observed in mice induced with hepatocarcinogenic NDEA for 5 weeks intraperitonal injection.

Materials and methods

Materials

N-Nitrosodiethylamine was purchased from Sigma-Aldrich (Tokyo, Japan). Normal saline was the product of PT. Widathra Bhakti (Pasuruan, Indonesia). This study used male Balb/c mice aged 6 weeks obtained from the animal laboratory, Faculty of Pharmacy, Universitas Airlangga.

Induction of NDEA in mice

All of the experimental procedures using animals had been approved by the Ethics Commission of Faculty of Veterinary, Universitas Airlangga. The mice were induced for liver disease by using NDEA diluted in normal saline. Mice were given NDEA intraperitonially at a dose of 25 mg/kgBW. The NDEA injection was given 5 times every 7 days for 5 weeks. The disease progress induced by NDEA was evaluated by weighing the mice body weight every week.

Preparation of mice organs

At the end of NDEA induction, the mice were then sacrificed and excised for evaluating their organs (heart, lungs, liver, spleen, and kidneys) including physical and morphological appearances. The organs including liver and spleen were excised and stored at -20˚C for further analysis. The organs were evaluated for the weight and morphological appearances. Moreover, the histopathology evaluations were also performed by haematoxylin-eosin staining for liver and spleen tissues.

Data Analysis

The results were presented as the mean ± SD. To determine the significant differences between data, a statistical analysis was carried out using the Oneway Analysis of Variance (ANOVA) method which was followed with the Honestly Significant Difference (HSD) post hoc test. The difference was statistically significant if the p value was <0.05.

Results

Body weight evaluation of mice induced with NDEA

To evaluate the results of NDEA induction, the mice induced by NDEA 25mg/kg per week were weighed every week and compared with mice injected with normal saline used as the control. The presence of weight loss in mice induced by hepatocarcinogens is one of parameters for cancer progress. The evaluation results of mice body weight can be seen in Figure 1. The NDEA-induced mice experienced weight loss while normal mice gained weight continuously. The results showed that there was a significant weight loss on the 29th day after 5 times NDEA induction. On the 31st day, the mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances.

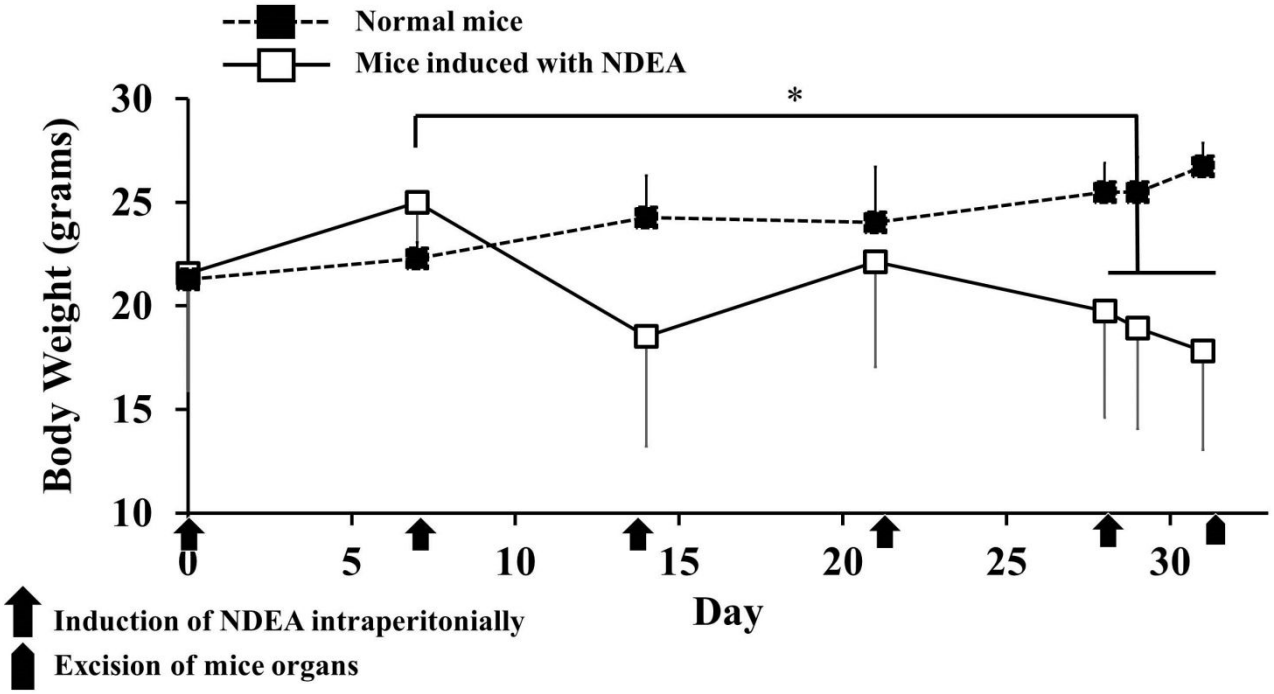

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). ***P*<0.05.

Physical appearances of mice organs

Based on observation of excised organs shown in Figure 2A-C, there were differences between organs specifically for liver and spleen of mice induced with normal saline and with NDEA for 5 weeks. In the control group, the morphological appearances of liver were shiny and bright red (Figure 2A). However, mice induced with NDEA had liver appearances with nodules and discoloration (Figure 2C). This suggests that NDEA induction for 5 weeks affects the liver cells, causes liver damage, and changes the external morphology of the liver of mice.

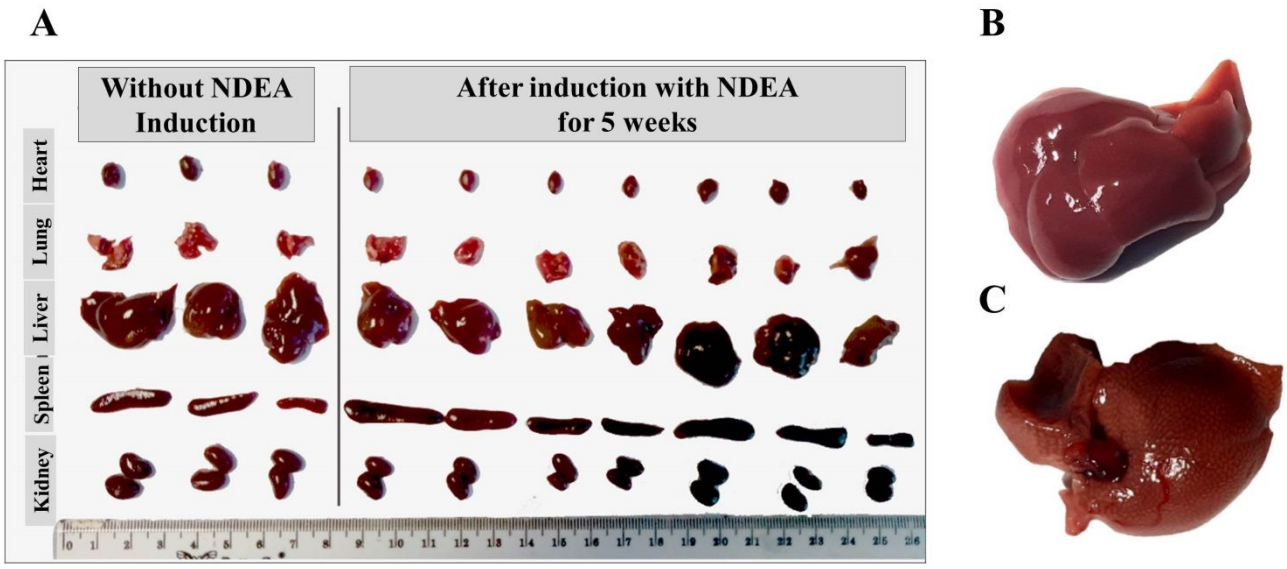

Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice.

Evaluation Weight of Mice Organ

The organ weights of mice in the control and NDEA-induction groups were evaluated to determine whether there were any significant differences on the physical weight during the induction. As it can be seen in Table 1, the liver in mice induced with NDEA was significantly relatively smaller than the control group (P<0.01), while the spleen were slightly smaller but no significant differences was observed (P >0.05).

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

Organ	Organ weights (mean ± SD)	
	Control	After NDEA Induction
Heart	0.11 ± 0.01 g	0.08 ± 0.03 g
Lungs	0.20 ± 0.04 g	0.32 ± 0.05 g
Liver	1.86 ± 0.13 g	0.97 ± 0.27 g
Spleen	0.23 ± 0.12 g	0.20 ± 0.12 g
Kidneys	0.40 ± 0.05 g	0.25 ± 0.06 g

Histopathological evaluations of liver tissue

According to the results as shown in Figure 3, the normal liver and spleen had regular architecture and cellular integrity with no fibrosis. After induction of NDEA, there were no malignancies observed in liver on spleen tissues in mice; however, there were single large fat droplets, alongside nuclei dislocation to the cell periphery that seemed to be macrovesicular steatosis. According to these results, there were lipidosis in liver and steatohepatitis observed for spleen tissue.

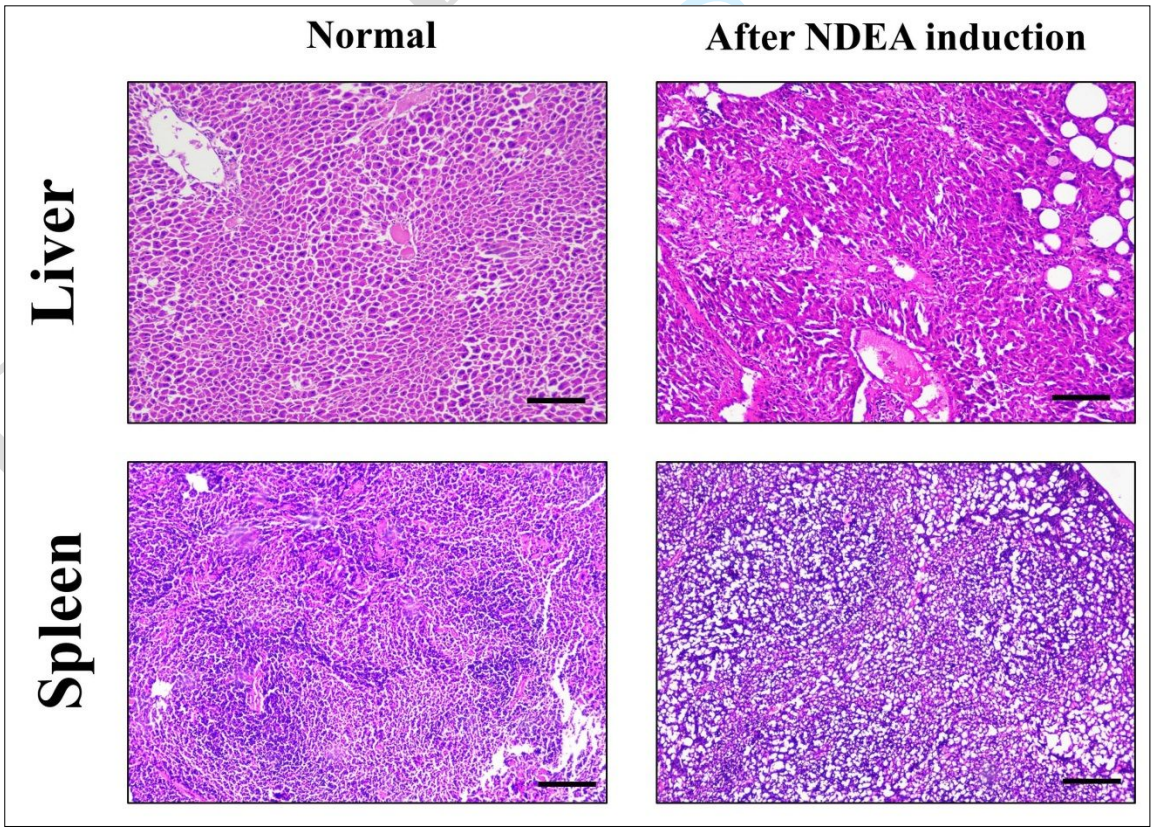

Figure 3: The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for 5 times. Scale bar= 100 µm.

Discussion

Making the ideal of animal models of liver disease with pathological analogous to liver disease in humans, especially for HCC cancer formation model both pathologically and biochemically is a challenge for researchers [20]. NDEA is a compound that is generally known to be mutagenic, teratogenic, and carcinogenic. Recent study reports that the use of NDEA as a hepatocarcinogen is known to have a strong ability and is able to induce primary liver cancer such as HCC which is at various stages of liver cirrhosis, besides that it can greatly simulate the histopathological evolution of clinical liver cancer [21].

It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents. NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [23]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [24]. However, in this study, there was no evaluation of food consumed by the mice during the experiments.

Based on the weight data for each organ shown in Table 1, it was known that the weight of liver organs in the treatment group decreased compared to control group. NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [25]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [26]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group.

NDEA induction for 5 weeks affects liver cells, causes liver damage, and changes the external morphology of the liver of mice. Previous studies report NDEA induction in mice causes a change in the structure of the liver in mice which is characterized by a reduction in size, discoloration, bleeding, scarring, and formation of nodule-like structures [27]. This is because NDEA is a toxic agent against the liver that can cause liver fibrosis [27], [28]. Fibrosis is formation of excess connective tissue, causing hardening and scar formation, in which about 20% of cancer cases are associated with chronic inflammation due to fibrosis, as found in liver cancer [29]. However, in this study, instead of malignancies, hepatic lipidosis and steatohepatitis were observed in mice liver and spleen after 5 weeks induction of NDEA. This indicates that the disease progress is still in the early stage of liver cancer diseases. It has been known that hepatic lipidosis is an early manisfestation of some other underlying conditions related to cancer, pancreatitis, and other liver problems [30]. Another study reports that rats induced with NDEA will show the appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis after 8 weeks of NDEA induction [22]. This early disease stage can be used for exploring preventive therapy of some drug compounds, such as for comparing the efficacy of drug delivery system. Lipid peroxidation and oxidative stress are dangerous to cells resulting in liver injury, which leads to liver fibrosis and cirrhosis or cancer. However, further biochemical investigation is required to definitely score the stage of liver disease after 5 weeks induction of NDEA.

Conclusion

Induction of NDEA in mice for 5 weeks results in hepatic lipidosis or fatty liver and steatohepatitis confirmed as the liver inflammation which may indicate the early stage of liver cancer disease, thus providing the potential use of NDEA for making animal models for the preventive cure of liver disease.

Acknowledgement

The author would like to thank to Alphania Rahniayu from Faculty of Medicine, Universitas Airlangga for her kind helps during the histopathology evaluation. This study was supported by a Preliminary Research on Excellence in Higher Education Institutions (Penelitian Dasar Unggulan Perguruan Tinggi, PDUPT) Grant Number AMD/E1/KP.PTNBH/2020 and 710/UN3/14/PT/2020 provided by the Ministry of Research and Technology-National Research and Innovation Agency of Republic of Indonesia.

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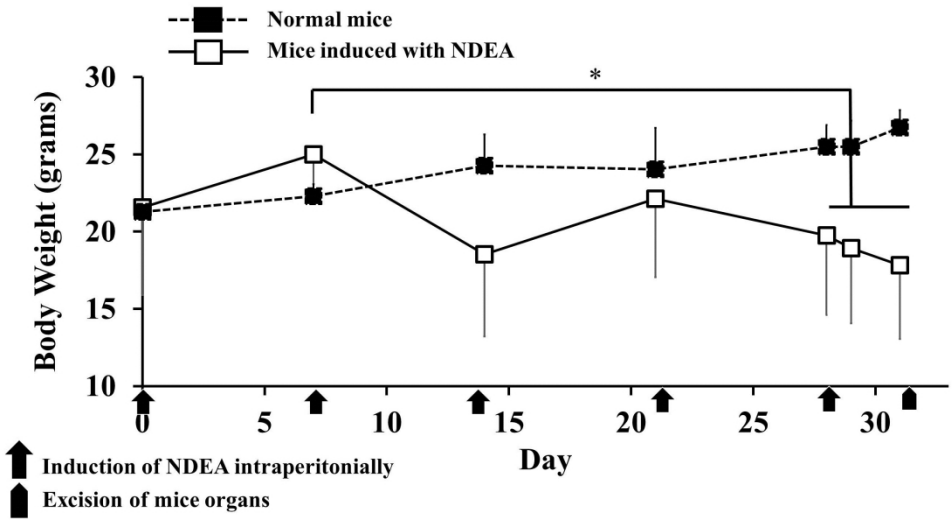

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). **P<0.05.

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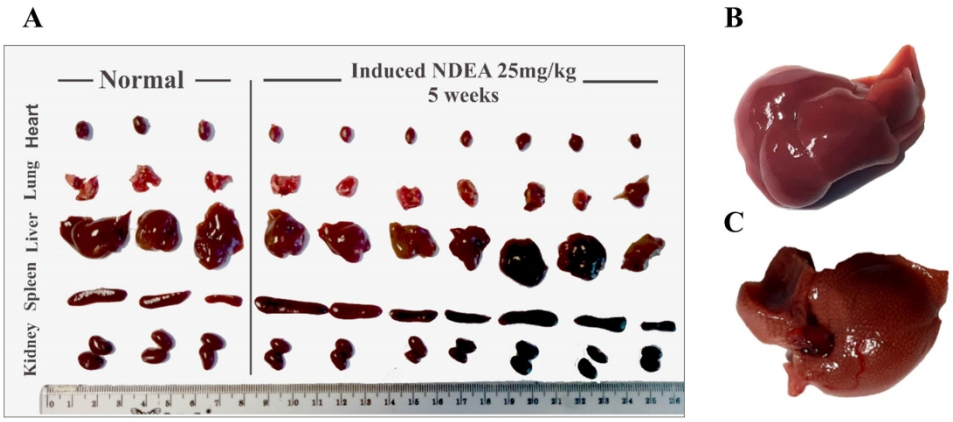

Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice.

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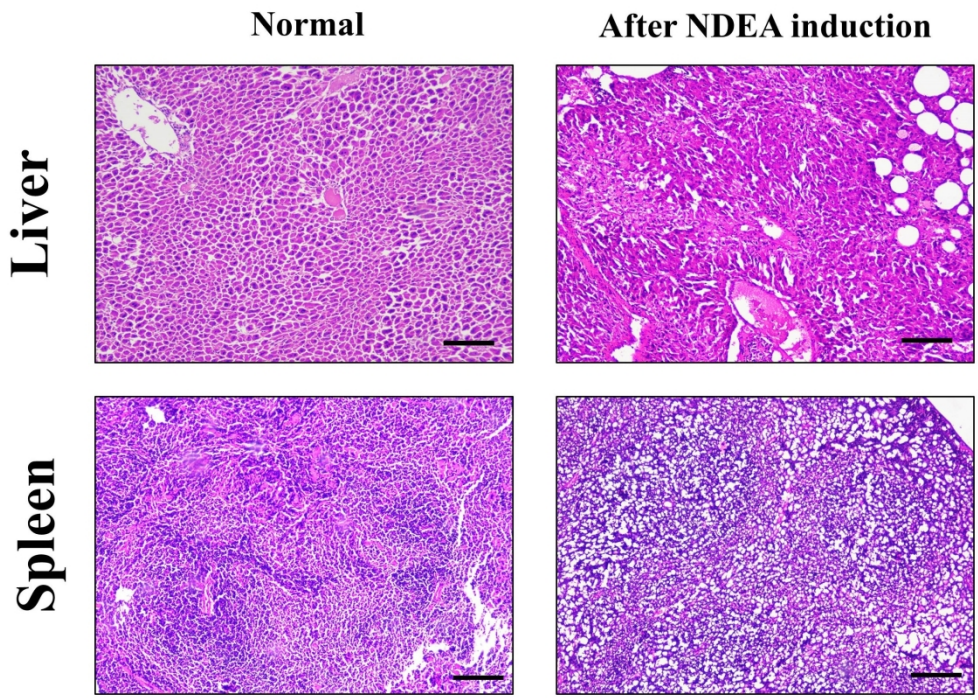

Figure 3: The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for 5 times. Scale bar= 100 µm.

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1 IDENTIFYING INFORMATION

Provide the date of submission and the title of your manuscript and the ID (in case you know it already). Give your full name and if you are NOT the corresponding author please check the box „no". Provide the full name and the initials of all co-authors and indicate, if applicable, the corresponding author.

2 THE WORK UNDER CONSIDERATION FOR PUBLICATION

This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you and/or any co-authors received, either directly or indirectly (via your institution), to enable the completion of the work. Checking „No" means that you and any co-authors did the work without receiving any financial support from any third party - that is, the work was supported by funds from the same institution that pays the salary and that institution did not receive third-party funds with which to pay you and/or any co-authors. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check „Yes".

3 RELEVANT FINANCIAL ACTIVITIES OUTSIDE THE SUBMITTED WORK

This section asks about any financial relationships you or any co-authors might have with entities in the scientific arena that could be perceived to influence, or that give the appearance of potentially influencing, what you wrote in the submitted work. You should disclose interactions with ANY entity that could be considered broadly relevant to the work. Report all sources of revenue paid (or promised to be paid) directly to you, any co-authors or related institutions on your behalf over the 36 months prior to submission of the work. This should include all monies from sources with relevance to the submitted work, not just monies from the entity that sponsored the research. Please note that your interactions with the work's sponsor that are outside the submitted work should also be listed here. If there is any question, it is usually better to disclose a relationship than not to do so. For grants you or any co-authors have received for work outside the submitted work, you should disclose support ONLY from entities that could be perceived to be affected financially by the published work, such as companies, or foundations supported by entities that could be perceived to have a financial stake in the outcome. Public funding sources, such as government agencies, charitable foundations or academic institutions, need not be disclosed. This section also asks about intellectual property such as patents and copyrights, whether pending, issued, licensed and/or receiving royalties.

4 RELATIONSHIPS NOT COVERED ABOVE

Use this section to report other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work.

SECTION 1.

IDENTIFYING INFORMATION - SUBMISSION

1. Effective Date (Day-Month-Year) 29-11-2020

2. Manuscript Title Animal model of liver disease in mice induced with n-nitrosodiethylamine

3. Manuscript Identifying Number (if you know it) -

IDENTIFYING INFORMATION - SUBMITTING AUTHOR

1. Given Name (First Name) Andang

2. Surname (Last Name) Miatmoko

3. Are you the corresponding author? Yes ☑ No ☐

Corresponding Author's Name -

IDENTIFYING INFORMATION - CO-AUTHOR

Please add all co-authors of your manuscript. Please ensure that you collected all relevant information of your co-authors correctly, since the submitting author is responsible for the accuracy and completeness of the submitted information.

THE WORK UNDER CONSIDERATION FOR PUBLICATION		
Last name	First name	Initials
Cahyani	Devy Maulidya	DMC
Miatmoko	Andang	AM
Hariawan	Berlian Sarasitha	BSH
Purwantari	Kusuma Eko	KEP
Sari	Retno	RS

SECTION 2.

THE WORK UNDER CONSIDERATION FOR PUBLICATION

Did you, a co-author or your institution at any time receive payment or services from a third party (government, commercial, private foundation, etc.) for any aspect of the submitted work (including but not limited to grants, data monitoring board, study design, manuscript preparation, statistical analysis, etc.)?

Are there any relevant conflicts of interest? Yes ☑ No ☐

If yes, please fill out the appropriate information below. Complete each row by checking "No" or providing the requested information.
Indicate the recipient of the payment or the service by adding the initials in the respective row.

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THE WORK UNDER CONSIDERATION FOR PUBLICATION						
Type	No	Recipient Initials	Money paid to you	Money paid to your Institution*	Name of Entity	Comments**
Grant	✔	AM	✔	☐	MENRISTEK-BRIN	Grant Number AMD/E1/KP.PTNBH/2020 and 710/UN3/14/PT/2020
	☐		☐	☐		
	☐		☐	☐		
Consulting fee or honorarium	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Support for travel to meetings for the study or other purposes	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Fees for participation in review activities such as data monitoring boards, statistical analysis, end point committees, and the like	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Payment for writing or reviewing the manuscript	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		

Provision of writing assistance, medicines, equipment, or administrative support	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Other	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		

* This means money that your institution received for your efforts on this study.
** Use this section to provide any needed explanation.

SECTION 3.

RELEVANT FINANCIAL ACTIVITIES OUTSIDE THE SUBMITTED WORK

Place a check mark in the appropriate boxes in the table to indicate whether you or any co-author have financial relationships (regardless of the amount of compensation) with entities as described in the instructions. Use one line for each entity; add as many lines as you need by clicking the „Add +“. You should report relationships that were present during the 36 months prior to submission. Indicate the person involved by adding the initials in the respective row.

Are there any relevant conflicts of interest? Yes ☐ No ☑

If yes, please fill out the appropriate information below. Complete each row by checking “No” or providing the requested information.
If the relevant information exceeds the available rows, please fill in an additional form and upload this as separate file to the submission system.

RELEVANT FINANCIAL ACTIVITIES OUTSIDE THE SUBMITTED WORK						
Type of Relationship	No	Initials	Money paid to You	Money paid to Your Institution*	Name of Entity	Comments**
Board Membership	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Consultancy	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Employment	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Expert testimony	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		

Grants/grants pending	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Payment for lectures including service on speakers bureaus	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Payment for manuscript preparation	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Patents (planned, pending or issued)	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Royalties	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Payment for development of educational presentations	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Stock/stock options	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		

Travel/accommo-dations/meeting expenses unrelated to activities listed	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
Other	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		
	☐		☐	☐		

* This means money that your institution received for your efforts.
** For example, if you report a consultancy above there is no need to report travel related to that consultancy on this line.

SECTION 4.

OTHER RELATIONSHIPS

Are there other relationships or activities that readers could perceive to have influenced, or that give the appearance of potentially influencing, what you wrote in the submitted work?

☑ No other relationships/conditions/circumstances that present a potential conflict of interest

☐ Yes, the following relationships/conditions/circumstances are present (explain below):

At the time of manuscript acceptance, journals will ask authors to confirm and, if necessary, update their disclosure statements. On occasion, journals may ask authors to disclose further information about reported relationships.

SOURCES

This form is based on the ICMJE Form for Disclosure of Potential Conflicts of Interest form (to be found here: http://www.icmje.org/conflicts-of-interest/).

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

Organ	Organ weights (mean ± SD)	
	Control	After NDEA Induction
Heart	0.11 ± 0.01 g	0.08 ± 0.03 g
Lungs	0.20 ± 0.04 g	0.32 ± 0.05 g
Liver	1.86 ± 0.13 g	0.97 ± 0.27 g
Spleen	0.23 ± 0.12 g	0.20 ± 0.12 g
Kidneys	0.40 ± 0.05 g	0.25 ± 0.06 g

Strictlyadhere to the given format.
Statements on Informed consent and Ethical approval may be removed **if not applicable**.

Acknowledgments

The author would like to thank to Alphania Rahniayu from Faculty of Medicine, Universitas Airlangga for her kind helps during the histopathology evaluation.

Research funding

This study was supported by a Preliminary Research on Excellence in Higher Education Institutions (Penelitian Dasar Unggulan Perguruan Tinggi, PDUPT) Grant Number AMD/E1/KP.PTNBH/2020 and 710/UN3/14/PT/2020 provided by the Ministry of Research and Technology-National Research and Innovation Agency of Republic of Indonesia.

Author contributions

All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

Competing interests

Authors state no conflict of interest.

Informed consent

Not applicable.

Ethical approval

The study protocol was approved by the Animal Care and Use Committee of the Faculty of Veterinary, Airlangga University with an Ethical Clearance No. 2.KE.022.02.2020.

Dear Editor,
Many thanks for the email. We really appreciate all comments to improve our manuscript. Below are the answers addressed for the reviewer's comment.

Reviewer: 1
Comments to the Author

The research describes the modeling for NDEA induced liver injury. The method and result have been described sufficiently. However, the model has been described elsewhere, e.g. doi: 10.1515/intox-2015-0001. It is important to make a good note in the manuscript about the novel finding in the study. The authors must focus on the liver disease if it is what they aim for. Following are the notes:

1. What is the new finding filling the gap in the field. Reports are showing the success of the model in other studies.
Answer:
In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer development. The reports on the use of NDEA for making liver inflammation itself are limited. For the future study, we would like to have some models for the treatment, including preventive and curative actions. The cancer models itself has been reported by induction of NDEA at a dose of 25 mg/Kg BW for 8 weeks, while the preventive mode is still a limited study.

We have revised the title into: "N-Nitrosodiethylamine induces liver inflammation in mice"

We have revised and added some sentences I page 1 line 6-10 as the following:
"For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction"

2. It is important to redefine the focus. In the title, it is written as liver disease, which varies in types. The background of the study implies the aim is liver cancer disease. The result showed many organ profiles. The histology data showed liver and spleen. This scrambled data and inconsistency should be adjusted to prevent confusion.
Answer:
In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer progression. So, we focused on liver and spleen as the target organs reflecting the inflammation model induced by NDEA intraperitoneal injection. However, we would like to show other organs to see whether there are any physical changes, which non-different morphologies were visually observed after the observation for lungs, heart, and kidney.

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“For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction”

We have revised and added some sentences into the background in line 27-50 as the following:
“The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumour initiation, growth, development, and metastasis [6]. Inflamation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte / macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].
In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic immune system, allowing cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].

Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a “secretome” that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].

The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC.”

We have also added sentences in line 52-54 as the following:
“Preventive care could be highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice is often used for animal model, especially for cancer research [11].”

We have also added sentences in line 68-69 as the following:

“NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy”

3. In the table 1 caption, the author should put the number of the sample (n) as the number of animal in 1 group, not all group. Further, the author should add the information of the value presentation of the result, whether it is mean ± SD or mean ± SEM, or else. For the normal group results, the result should be added by SEM or SD value compared to the NDEA group.

Answer:

Many thanks for the comments. We have added the sampel number in each figure or table legends, as the following:

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). ***P*<0.05..

Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice.

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

We have also added the “Organ weights (mean ± SD)” in the column title of Table 1.

In line 91, we have revised the sentence as the following:
“The results were presented as the mean ± SD”

4. It is also important to include the Error/ Deviation bar for the Normal group line in figure 1 and the statistical mark for the comparison to the normal group. It is assumed that the normal group line was made by the mean of some samples.

Answer:

We have revised the figure 1 and Table 1 by adding standard deviation of measurement, especially for the control or normal group.

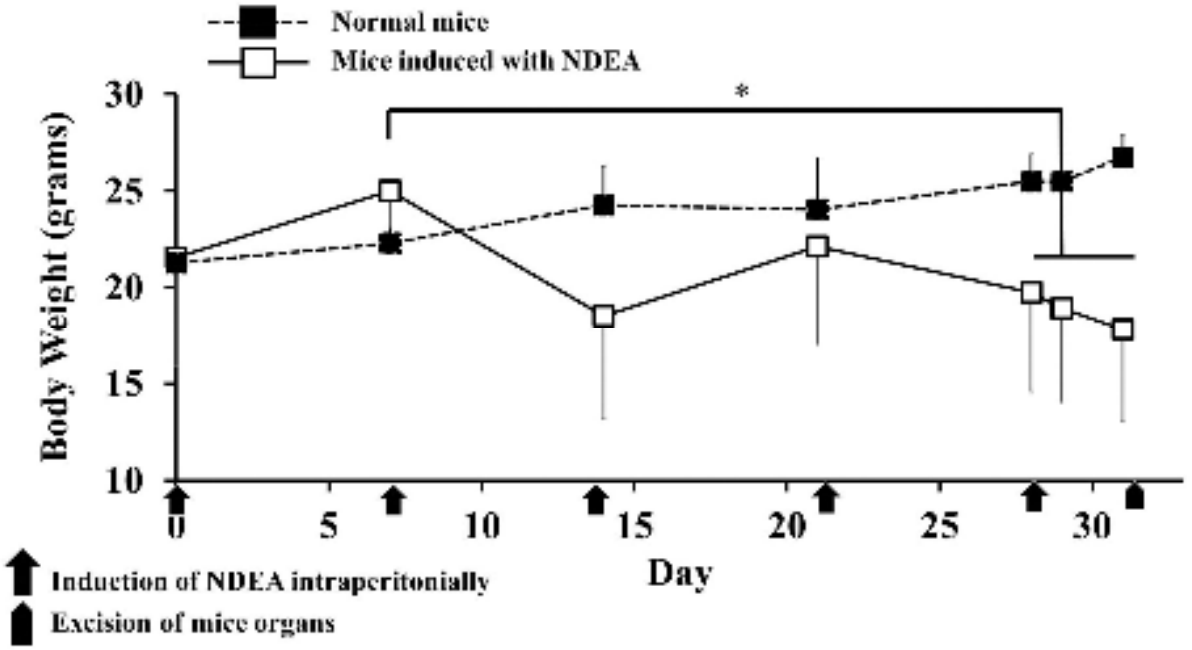

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25mg/kg intraperitonially once a week for 5 times and mice were then sacrificed at day 31 (n = 7). ***P*<0.05.

Table 1. Evaluation of mice organ weights in the control group (n=3) to the NDEA-induced group with a dose of 25mg / kg 5 times then mice were sacrificed and excised for evaluating their organ (n = 7).

Organ	Organ weights (mean ± SD)	
	Normal	After NDEA Induction
Heart	0.11 ± 0.01 g	0.08 ± 0.03 g
Lungs	0.20 ± 0.04 g	0.32 ± 0.05 g
Liver	1.86 ± 0.13 g	0.97 ± 0.27 g
Spleen	0.23 ± 0.12 g	0.20 ± 0.12 g
Kidney	0.40 ± 0.05 g	0.25 ± 0.06 g

5. The author explains about cancer-related cachexia represented by the weight loss data. This is irrelevant since there is no evidence of cancer formation in the present finding.
Answer:
According to the previous study, induction of NDEA resulted in lesser food intake of mice than the normal group causing the weight losses. We have revised and added discussion about this in line 143-147 as the following:
"It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease

as target for preventive cure of naticancer agents. NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [23]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [24]. However, in this study, there was no evaluation of food consumed by the mice during the experiments..”

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, “The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis,” *Heliyon*, vol. 5, no. November 2018, p. e01769, 2019.

[23] N. S. Thomas, K. George, S. Arivalagan, V. Mani, A. I. Siddique, and N. Namasivayam, “The in vivo antineoplastic and therapeutic efficacy of troxerutin on rat preneoplastic liver: biochemical, histological and cellular aspects,” *Eur. J. Nutr.*, vol. 56, no. 7, pp. 2353–2366, 2016.

[24] V. Rajesh and P. Perumal, “Chemopreventive and antioxidant activity by Smilax zeylanica leaf extract against N-nitrosodiethylamine induced hepatocarcinogenesis in wistar albino rats,” *Orient. Pharm. Exp. Med.*, vol. 14, no. 2, pp. 111–126, 2014.

6. It is not stated elsewhere whether the method is purposively made as short term induction. It should be defined whether the term of treatment should be in purpose to describe the targeted features in the model.

Answer:

In this study, the short term induction refers to shorter periods of NDEA induction, which was 5 weeks, than the previous study that stated 8 weeks induction of NDEA produced liver cancer. According to this comment, we have revised the definition of short term by changing “short term: with “after 5 weeks”, as the following:

Page 1 Line 9: “induced with hepatocarcinogenic NDEA for 5 weeks induction”

Line 69-70: “Thus, this study aimed to evaluate the liver disease model observed in mice induced with hepatocarcinogenic NDEA for 5 weeks intraperitonal injection.”

Line 145-146: “NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1).”

Line 162-163: “However, in this study, instead of malignancies, hepatic lipidosis and steatohepatitis were observed in mice liver and spleen after 5 weeks induction of NDEA”

Line 170: “stage of liver disease after 5 weeks induction of NDEA.”

Line 172: “Induction of NDEA in mice for 5 weeks”

7. Decrease of mice organ is possible because NDEA is also able to induce tumors in various organs such as the lungs, liver, esophagus, kidneys, stomach, intestines, and nervous system. This explanation is somehow made confusing. Again, there is no evidence of cancer occurring in the model. The author should make a logical explanation regarding the change in organ weight.

Answer:

Thank you for the comment. In this study, there were no significant different of organ weights of heart, lungs, kidney, and spleen between control and NDEA induction group, however, after NDEA induction, the liver weight was significantly decreased.

We have revised by deleting those statements in the discussion section, and revised the discussion in line 152-156 as the following:

”NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [25]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [26]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group.”

[25] G. Mittal, A. P. S. Brar, and G. Soni, “Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: Biochemical and histopathological effects,” *Pharmacol. Reports*, vol. 58, no. 3, pp. 413–419, 2006.

[26] R. C. Cattley and J. M. Cullen, *Liver and Gall Bladder*. 2013. Chapter 45: *Liver and Gall Bladder*. In W.M. Haschek, C.G.Rousseaux, M.A. Wallig, B.Bolon, R. Ochoa & B.M. Wahler (Eds). Haschek & Rouseaux's Handbook of Toxicology Pathology (3rd). Boston: Academic Press

8. There is no relevance in featuring non-liver organ’s weight. There is no correlative explanation of why it is done.

Answer:

In this study, we excised all organs from the mice of the control and NDEA-induced groups to compare whether there were changes in physical appearances and organ weights, which may could be used for analyzing the effect of NDEA inducing inflammation to mice organs. It because of the ability of NDEA for inducing tumors in various organs such as the lungs, liver, esophagus, kidneys, stomach, intestines, and nervous system. However, there was significant different in liver visual appearance and liver weight as well as confirmed by histology evaluation. .

We have revised and added sentences in line 150-155 in the paragraph as the following:

“Based on the weight data for each organ shown in Table 1, it was known that the weight of liver organs in the treatment group decreased compared to control group. NDEA administration causes liver degeneration as evidenced by a significant reduction in liver weight index [25]. This relative liver weight assessment can be used as an evaluation in diagnosing liver disease characterized by changes in liver size. Liver weight loss generally reflects loss of function associated with atrophy or hepatocellular injury [26]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group”

[25] G. Mittal, A. P. S. Brar, and G. Soni, “Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: Biochemical and histopathological effects,” *Pharmacol. Reports*, vol. 58, no. 3, pp. 413–419, 2006.

[26] R. C. Cattley and J. M. Cullen, *Liver and Gall Bladder*. 2013. Chapter 45: *Liver and Gall Bladder*. In W.M. Haschek, C.G.Rousseaux, M.A. Wallig, B.Bolon, R. Ochoa & B.M. Wahler (Eds). Haschek & Rouseaux's Handbook of Toxicology Pathology (3rd). Boston: Academic Press

Reviewer: 2

Comments to the Author

1. Cohesion between sentences not good enough. Please proofread this paper.

Answer:

We have proofread the manuscript.

2. **Please look at figure 2, "The visual observation of normal liver (B) and the liver after NDEA induction (B) of mice." Is it true?**

Answer:

We have revised the figure legend as the following:

"Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for 5 times, n=7, (A). The visual observation of normal liver (B) and the liver after NDEA induction (C) of mice."

3. **NDEA is well known to induce hepatotoxicity in the preclinic study, is it any different valuable information to strengthen this study?**

In this study, we proposed the use of NDEA for making an animal model with liver inflammation disease, which is purposed as the model for an early stage of liver cancer progression. So, we focused on liver and spleen as the target organs reflecting the inflammation model induced by NDEA intraperitoneal injection. However, we would like to show other organs to see whether there are any physical changes, which non-different morphologies were visually observed after the observation for lungs, heart, and kidney.

We have revised the title into: "N-Nitrosodiethylamine induces liver inflammation in mice"

We have revised and added some sentences I page 1 line 6-10 as the following:

"For designing early treatment for liver cancer, it is important to prepare an animal model to evaluate cancer prevention treatment by using inflammation disease. The hepatocarcinogenic N-Nitrosodiethylamine (NDEA) has been reportedly able to produce free radicals that cause liver inflammation leading to liver carcinoma. This study aimed to evaluate the inflammation disease model of mice induced with hepatocarcinogenic NDEA for 5 weeks induction"

We have revised and added some sentences into the background in line 27-50 as the following:

"The cancer progression includes initiation, inflammation, and cancer progression. Inflammation is a predisposing factor in cancer development and promotes the stage of tumorigenesis. Inflammation promotes the incidence of tumour initiation, growth, development, and metastasis [6]. Inflamation is considered as an important factor during cancer progression. Local inflammation in liver may be driven by infiltrating immune cells such as monocyte / macrophages, T lymphocytes, and neutrophils. Thus, inflammation is also caused by nonparenchymal cells such as kupffer cells, dendritic cells, liver sinusoidal cell, and hepatic stellate cells [7].

In cancer treatment, the early stage of cancer progression should determine the success of therapy. Inflammation in liver could highly lead to liver carcinoma. Chronic liver inflammation damages hepatic epithelial cells, including hepatocytes and biliaryepithelial cells. Because liver has a high regenerative capacity, this damage induces substantial cell proliferation. Simultaneously, inflammation induces reactive oxygen species (ROS) and deoxyribonucleic acid (DNA) damage, increasing the frequency of genomic DNA mutations. When the high rate of cell proliferation is coupled with DNA mutation, the incidence of malignant transformation increases. Further, chronic inflammation induces changes in the hepatic immune system, allowing cancer cells to easily evade immune surveillance. In most cases, chronic liver inflammation and the

resultant cirrhotic microenvironment promote the initiation and progression of HCC and CCA [8].
Local inflammation in hepatic tissue is driven by infiltrating immune cells (monocytes/macrophages, T lymphocytes, and neutrophils) and also by resident liver nonparenchymal cells [Kupffer cells, dendritic cells, liver sinusoidal cells, and hepatic stellate cells (HSCs)]. In a complex organ such as the liver, different cell types can secrete diverse cytokines/chemokines, and the resulting cocktail constitutes a "secretome" that leads to immunomodulation that manifests as an acute or chronic inflammatory response. Chronic inflammation acts as a favorable preneoplastic setting [7].
The acute inflammatory response occurs immediately or in minutes, hours, or days following injury. Normally, this is a physiologically beneficial response that helps in clearing injured hepatocytes and leads to wound healing. When this process fails, an overdrive of immune cells occurs that perpetuates as chronic inflammation [9]. As the name suggests, chronic inflammation is a prolonged progressive process lasting for months that tilts the homeostasis more toward damage than toward healing. In liver, chronic inflammation eventually sets the stage for progression toward cirrhosis and eventually to HCC."

We have also added sentences in line 52-54 as the following:
"Preventive care could be highly help the disease into good prognosis and reducing the mortality rate. Moreover, the key success for cancer therapeutic highly depends on the early stage of cancer progression. The mice is often used for animal model, especially for cancer research [11]."

We have also added sentences in line 68-69 as the following:
"NDEA is known to induce damage to the liver. It is useful in the treatment of cancer since the early stages of cancer development are an essential stage in determining the success of therapy"

4. There is a very weak to relate between weight loss and hepatocarcinogens.
Answer:
According to the previous study, induction of NDEA resulted in lesser food intake of mice than the normal group causing the weight losses. We have revised and added discussion about this in line 143-149 as the following:

"It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents. NDEA induction at a dose of 25mg/kgBW for 5 weeks showed that there were significant weight losses as shown in (Figure 1). In the previous study, administration of NDEA reduces the body weights in which the mice become lesser in food intake [23]. The weight loss observed during NDEA induction in mice is probably due to decreased liver function and nutritional deficiencies which may be due to reduced food intake [24]. However, in this study, there was no evaluation of food consumed by the mice during the experiments."

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, "The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis," *Heliyon*, vol. 5, no. November 2018, p. e01769, 2019.
[23] N. S. Thomas, K. George, S. Arivalagan, V. Mani, A. I. Siddique, and N. Namasivayam, "The in vivo antineoplastic and therapeutic efficacy of troxerutin on rat preneoplastic liver: biochemical, histological and

cellular aspects," *Eur. J. Nutr.*, vol. 56, no. 7, pp. 2353–2366, 2016.
[24] V. Rajesh and P. Perumal, "Chemopreventive and antioxidant activity by Smilax zeylanica leaf extract against N-nitrosodiethylamine induced hepatocarcinogenesis in wistar albino rats," *Orient. Pharm. Exp. Med.*, vol. 14, no. 2, pp. 111–126, 2014.

5. **There is very lack of evidence in this result study that NDEA induced hepatocarcinogens, pathology anatomy in surrounding tissue assessment must be provided to support this statement, not only morphology and the evaluation.**

Answer:

In this study, we have evaluated the histopathology evaluations by haematoxylin-eosin staining for liver and spleen tissues. According to the results as shown in Figure 3, the normal liver and spleen have regular architecture and cellular integrity with no fibrosis. After induction of NDEA, there were no malignancies observed in liver on spleen tissues in mice; however, there were single large fat droplets, alongside nuclei dislocation to the cell periphery, seems to be macrovesicular steatosis. According to these results, there were lipidosis in liver and steatohepatitis observed for spleen tissue.

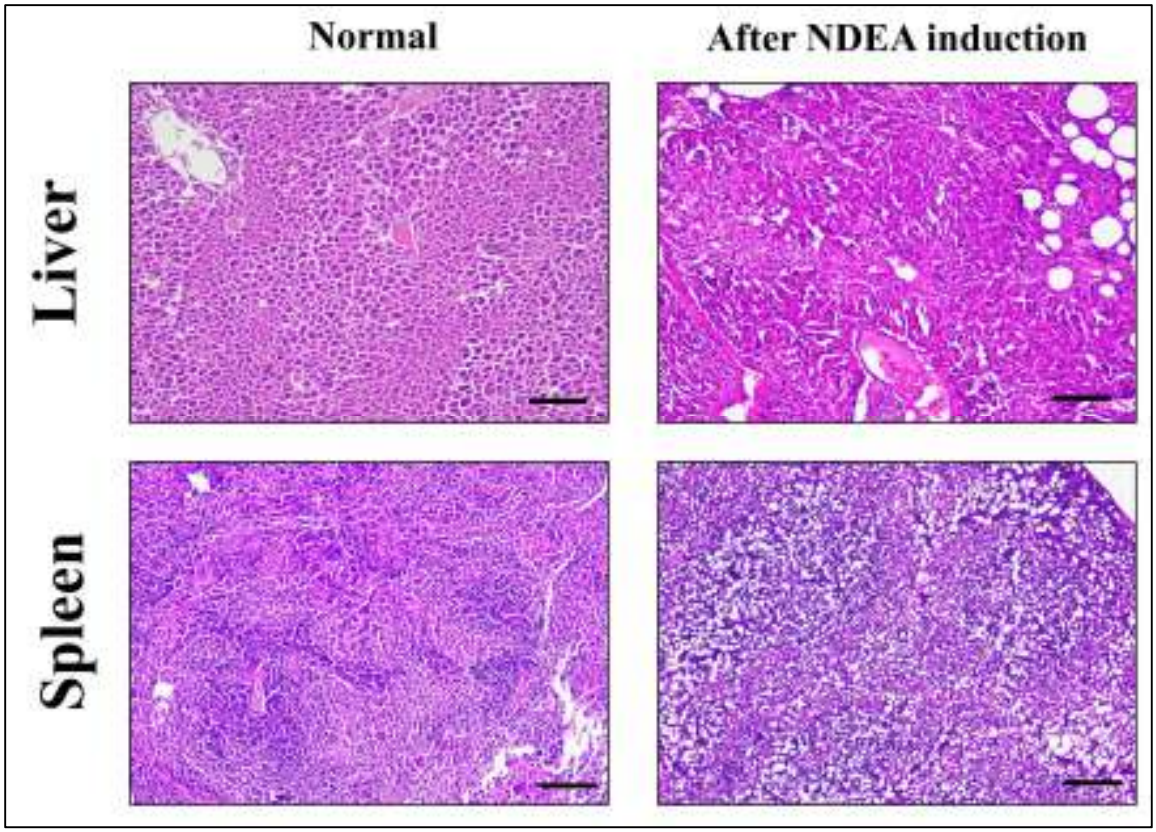

Figure 3: The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for 5 times. Scale bar= 100 µm.

6. **Give a logical explanation about the length of NDEA intervention that can produce a carcinogenic cell. How many replications in this study?**

Answer:

According to the previous study, the induction of NDEA for 8 weeks resulted in hepatocellular carcinoma indicated by enlarged hyperchromatic nucleus of hepatocytes in liver tissue and

scattered mitosis [22]. However, to produce an animal model for an early stage of cancer or preventive cure of cancer, in this study, NDEA was induced for 5 weeks and as indicated by the weight loss, the inflammation process has occurred.
We have added some discussion in line 143-145 as the following:
“It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered mitosis in liver tissue [22]. In this study, NDEA was used to produce an animal model for inflammation liver disease as target for preventive cure of naticancer agents.”
In this study, there were 7 mice in NDEA induction group and 3 mice for the control group.

[22] S. A. Ali, N. A. Ibrahim, M. M. D. Mohammed, S. El-hawary, and E. A. Refaat, “The potential chemo preventive effect of ursolic acid isolated from Paulownia tomentosa , against N-diethylnitrosamine : initiated and promoted hepatocarcinogenesis,” *Heliyon* vol. 5, no. May, 2019.