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 five weeks induction.

Methods: The BALB-c mice were induced with NDEA 25 mg/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 five 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.

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Keywords: cancer; 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 *cholangiocyte* 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 tumor initiation, growth, development, and metastasis [6]. Inflammation 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.

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 [15]. 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 [16]. 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 [17]. 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 five 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 six 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 five times every seven days for five 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 hematoxylin-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 25 mg/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 five times NDEA induction. On the 31st day, the mice were then sacrificed and excised for evaluating their organs including physical and morphological appearances.

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 five 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 five weeks affects the liver cells, causes liver damage, and changes the external morphology of the liver 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 ($p>0.05$).

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.

Discussion

Making the ideal of animal models of liver disease with pathological analogous to liver disease in humans,

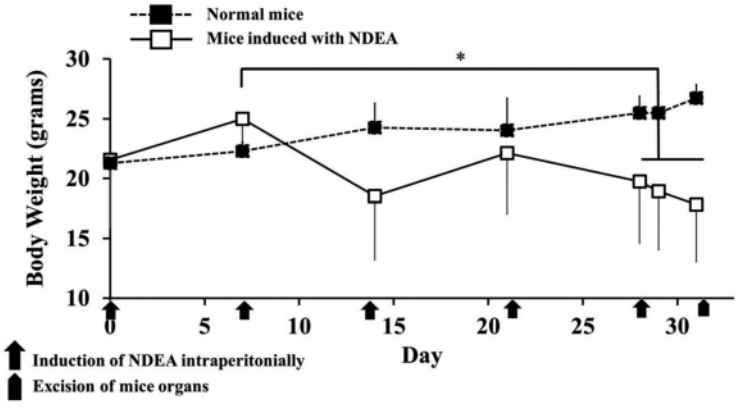

Figure 1: The mean of normal mice body weights (n=3) compared to mice induced with NDEA at a dose of 25 mg/kgBW intraperitonially once a week for five times and mice were then sacrificed at day 31 (n=7). **p<0.05.

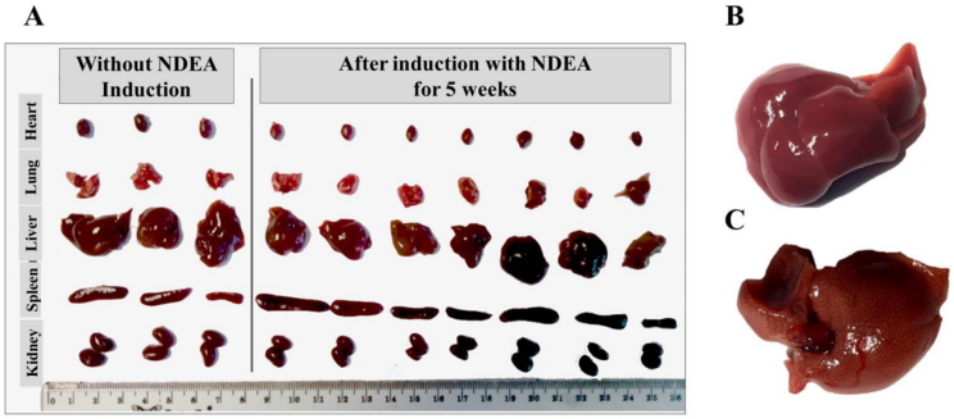

Figure 2: The physical appearances of mice organs including heart, lungs, liver, spleen, and kidneys from normal group treated with normal saline (n=3) and the NDEA-induced mice at a dose of 25 mg NDEA/kgBW once a week for five times, n=7.
(A) The visual observation of normal liver (B) and the liver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

especially for HCC cancer formation model both pathologically and biochemically is a challenge for researchers [18]. 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 [19].

It has been reported previously that induction of NDEA for 8 weeks resulted in hepatocellular carcinoma as indicated by enlarged hyperchromatic nucleus and scattered

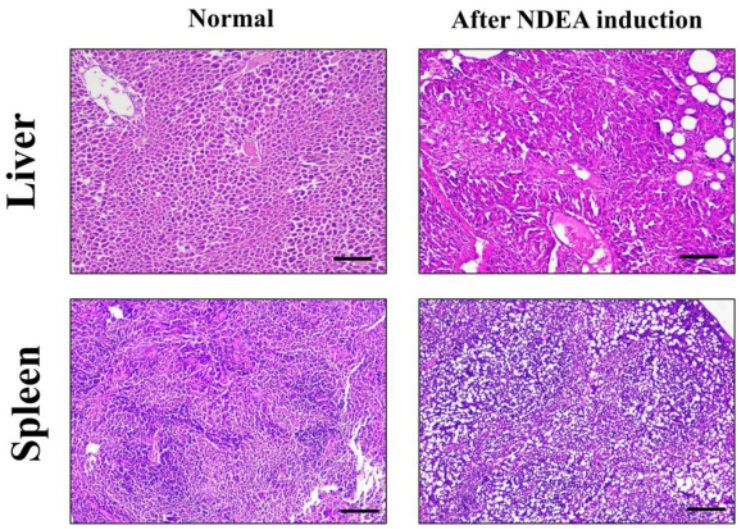

Figure 3: The histopathology photomicrographs of mice liver and spleen tissues stained with hematoxylin-eosin taken from specimens of normal mice and mice intraperitonially injected with NDEA at a dose of 25 mg NDEA/kgBW once a week for five times. Scale bar=100 µm.

mitosis in liver tissue [20]. 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 25 mg/kgBW for five 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 [21]. 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 [22]. 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 [23]. 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 [24]. However, in this study, the mice induced with NDEA showed no differences in the lymph weight compared to control group.

NDEA induction for five 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 [25]. This is because NDEA is a toxic agent against the liver that can cause liver fibrosis [25, 26]. 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 [27]. However, in this study, instead of malignancies, hepatic lipidosis and steatohepatitis were observed in mice liver and spleen after five 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 manifestation of some other underlying conditions related to cancer, pancreatitis, and other liver problems [28]. Another study reports that rats induced with NDEA will show the appearance of hepatocellular carcinoma with enlarged hyperchromatic nuclei and scattered mitosis after eight weeks of NDEA induction [20]. 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 five weeks induction of NDEA.

Conclusion

Induction of NDEA in mice for five 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.

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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.

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