Journal of
Biological Sciences

The Effect of Neem (*Azadirachta indica*) Aqueous Extract and Dietary Selenium on Distribution of Selenium in Liver Tissue During Hepatocarcinogenesis

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Abstract: This study aimed to determine the distribution of selenium in the liver of rats during hepatocarcinogenesis when neem aqueous extract and dietary selenium was supplemented. The selenium distribution of the 9 diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF) induced hepatocarcinogenesis in *Sprague dawley* male rats as well as the other 9 normal rats, which were supplemented with 5% (w/v) neem leaves aqueous extract, 1.85 mg L^{-1} sodium selenate and tap water *ad libitum* according to the treatment groups for 10 weeks, were assessed with energy filter transmission electron microscope (EFTEM) by electron spectroscopic imaging (ESI)-3 window power law method. The result showed that the selenium distribution mean score in normal control group was the lowest and cancerous control group was the highest. However, the selenium distribution mean score in normal supplemented with 5% (w/v) neem leaves aqueous extract increased about 26% (p<0.05) compared to normal control group. This study suggested that neem leaves aqueous extract may be a potential primary chemopreventive agent rather than as secondary chemopreventive agent.

Key words: Antioxidant element, elemental mapping, hepatocarcinogenesis

INTRODUCTION

Liver cancer, or hepatocellular carcinoma (HCC) represents more than 5% of all cancers in the world and estimated number of cancer-related deaths exceeds 500,000 per year[1]. It has high incidence in Southeast Asia and Africa[2]. The common etiologic associations of liver cancer include, HBV or HCV infection, chronic liver disease (alcohol and chemicals abuse) and specific hepatocarcinogens in food (primarily aflatoxins).

The neem, *Azadirachta indica*, is a member of the *Meliaceae* (mahogany) family. Neem has been extensively used in India as traditional Ayurvedic and folklore medicine for the treatment of various diseases. The extract from all parts of the tree, including the bark, leaves, fruits, oil and root has been reported to be anti-inflammatory, antipyretic and hypoglycaemic[3], also exhibits antimicrobial and anticancerous properties[4-7] and also as a contraceptive agent in male[8]. In this study, neem leaves extract was used to treat hepatocarcinogenesis induced rats and the selenium distribution in the liver tissue was assessed with comparison to sodium selenate treated group. The objective of this study was to determine the distribution of selenium in liver tissue during hepatocarcinogenesis in rats when neem leaves aqueous extract and dietary selenium is supplemented.

MATERIALS AND METHODS

***In vivo* bioassay and transmission electron microscope tissue processing:** In this experiment, 18 male with weight 200- 250 g and 6-8 weeks old *Sprague dawley* rats were purchased from the animal colony unit, University Putra Malaysia (UPM). These rats were maintained at animal house of Faculty of Medicine and Health Sciences, Universiti Putra Malaysia for 15 weeks and acclimatized for at least a week before use. They were kept in separate cages in a ventilated room with equal periods of daylight and darkness with temperature (32±2°C). Rat feed and water *ad libitum* were given to these rats daily. Each cage was cleaned every week and bedded with wood chip for urine absorption.

The neem (*Azadirachta indica*) leaves were collected from the Horticulture Department (UPM). The aqueous extract of neem leaves was prepared from the

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modification of green tea extraction according to Conney *et al.*[9]. In this experiment according previous research, 5% (w/v) of leave extract was used.

Protocol of the hepatocarcinogenesis was basically according to Solt and Farber method[10]. The method was modified, as the rats did not undergo the partial hepatectomy (selective pressure) stage. In this study, the rats were divided to six groups and each group consists of 6 rats. Rats in Group 1, 2 and 3 were injected 200 mg kg^{-1} b.w. diethylnitrosamine (DEN) intraperitoneally as an initiator to hepatocarcinogenesis and after 2 weeks, the rat feed which was mixed with 2-acetylaminofluorene (2-AAF) was given to these rats as promoter of hepatocarcinogenesis. On the other hand, rats in Group 4, 5 and 6 served as normal control and were injected with corn oil intraperitoneally.

At first week, treatment with 5.0% (w/v) neem (*Azadirachta indica*) leaves aqueous extract were given to the rats in Group 2 and 5 as a substitute to water. Rats in Group 3 and 6 were supplemented with 1.85 mg L^{-1} sodium selenate (Na$_2$SeO$_4$) solution. However, rats in Group 1 and 4 did not receive any treatment.

After 10 weeks of treatment, the rats were sacrificed under diethylether anesthesia. The livers of these rats were removed and weighted. The liver was washed in ice-cold 0.9% sodium chloride solution as soon as after removed from the animal.

For the transmission electron microscopy work, the liver was cut into small sections measured 1 mm^3 in size and quickly fixed in 4% buffered glutaraldehyde for overnight at 4°C. Then, the tissues were rinsed repeatedly in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide for 2 h, followed by ascending acetone dehydration. Finally, the tissue was embedded in agar 100 resin.

Elemental analysis: To reveal the intracellular distribution of selenium, ultrathin sections (approximately 60 nm thickness) of embedded liver tissues were examined without lead citrate counterstaining in the energy filter transmission electron microscope LEO 912AB equipped with an tungsten source and operated at 120 kV. The LEO 912AB features a Koehler-type illumination system and an in column omega type electron energy filter.

Due to the Koehler type illumination system of the microscope, the irradiation can reproducibly be adjusted. Setting the emission current to 4 μA and the condenser system to 2.5 mrad resulted in a dose rate of 6.0x10^4 e$^-$ (nm^2 s)$^{-1}$. Exposure time was 1s for each image. The entrance aperture of the spectrometer was set at 1.5 mm. The spectrometer slit width was set to 50 eV and the primary magnification to 20 000x. All images were corrected for the camera offset and gain variations. After

averaging 2x2 pixels, the effective pixel size on the resulting 512x512 images was 1.4 nm. For ESI acquisition, the three window power law method was used[11].

Statistical analysis: Mann-Whitney test (non-parametric test) were used to analyze the elemental distribution data, to knows the significant difference between group by using the SPSS software (11.0).

RESULTS AND DISCUSSION

The selenium distribution mean score in the hepatocytes of cancerous control was the highest among the 6 treatment groups. This finding supports the findings of several workers who have observed enhancement in the concentration of selenium in cancerous breast and other tissue using other research methods[12-17]. Ng *et al.*[18] noted that the trace element concentrations in malignant tissues were elevated as compared to corresponding levels for normal tissues.

The selenium distribution mean scores in the hepatocytes of cancer+neem group and cancer+selenium group were slightly lower (p>0.05) than the cancerous control. However, the selenium distribution mean scores in the hepatocytes of normal+neem group (p<0.05) and normal+selenium group were higher than the normal control group and their score were almost the same with the 3 cancerous groups (Fig. 1).
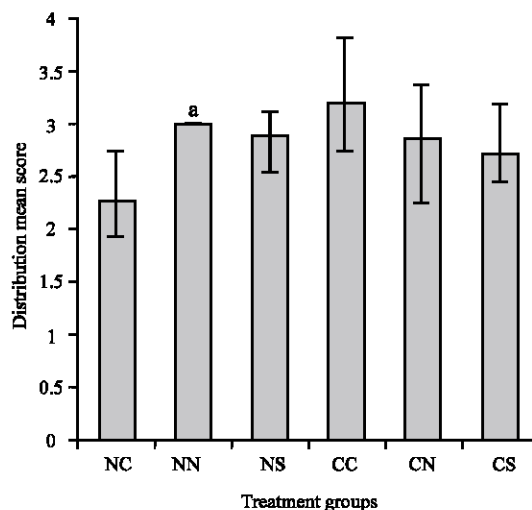

Fig. 1: Selenium distribution mean score in the hepatocytes of various treatment groups. NC=normal without treatment, NN=normal+neem, NS=normal+selenium, CC=cancerous without treatment, CN=cancer+neem, CS=cnacer+selenium, a: significant (p<0.05) with normal without treatment (NC)
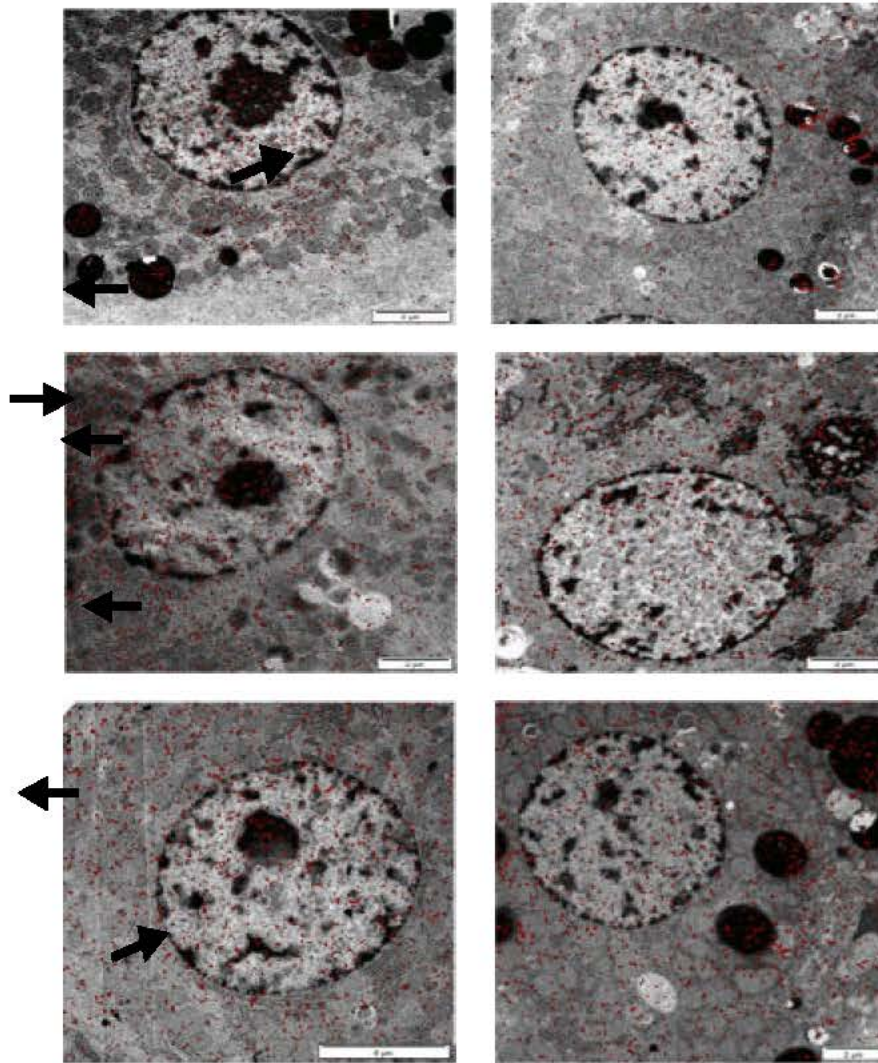
Fig. 2: Elemental analysis of the hepatocytes from different treatment groups with EFTEM. All the images shown are selenium map, recorded with the Se $L_{2,3}$ ionization edge (three-window power law method). Scale bar indicated 0.2 µm

a. Cancerous control: Some of the selenium is detected in the electron dense granules (arrowheads) and most is located mainly on the ribosome and rough endoplasmic reticulum (rER)

b. Cancer+Neem: The selenium is distributed in the cytoplasm and nucleus (arrowheads)

c. Cancer+selenium: The selenium is widely distributed in the cytoplasm and nucleus. (arrowheads)

d. Normal control: The selenium detected is very little and in the cytoplasm, some selenium is also detected in the electron dense granules (arrowheads)

e. Normal+Neem: The selenium is widely distributed in the cytoplasm and nucleus. In the cytoplasm, it is detected on the ribosome (arrowheads)

f. Normal+selenium: The selenium is distributed in the cytoplasm and nucleus. Lots of the selenium was detected in the electron dense granules (arrowheads)

Selenium was detected mainly at the euchromatin and at the nucleoli (Fig. 2). On the other hand, in the cytoplasm, selenium was detected mainly on the ribosome as well as the rough endoplasmic reticulum (rER). It was observed that in some of the micrographs, especially for the cancerous control group, normal control group and normal+selenium group, there were plenty of electron-dense granules observed in the cytoplasm of the hepatocytes. These electron-dense granules were suspected to be liver enzyme granules or glycogen

granules. However, they are high possibility to be liver enzyme granules. The evidences are that there were a lot of selenium detected on the ribosome and rough endoplasmic reticulum (rER), which are the main sites for protein synthesis and this may suggest the cells were actively synthesis protein with selenium components. Therefore, the selenium detected in those granules may suggest the presence of selenium compounds or certain liver enzymes with selenium component, such as the glutathione peroxidase (GPx), SeP, SeW and others. However, it is undeniable that those granules may also be glycogen granules as liver also involves in metabolic functions, e.g. glycogen synthesis, gluconeogenesis and storage of glycogen[19].

In this study the effect of neem (*Azadirachta indica*) leaves extract and sodium selenate supplementation on the selenium distribution in rat liver tissue during hepatocarcinogenesis, the result showed that the two supplementations did not significantly affect the selenium distribution in the liver tissue.

Based on the findings of the present study, it may be suggested that neem (*Azadirachta indica*) leaves extract and sodium selenate may be potential primary chemopreventive agent but does not potentiate as secondary chemopreventive agent. However, final conclusion can not be made at this stage as the results presented in this study were very subjective. Unless, quantification of the elements in the cells is able to be carried out or alternative method, such as the atomic absorbance spectrometry (AAS), will be made significant.

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