UNIVERSITI PUTRA MALAYSIA

EFFECTS OF Morinda citrifolia ON N-METHYL N-NITROSOUREA-INDUCED PERIPHERAL T CELL NON-HODGKIN’S LYMPHOMA IN SPRAGUE DAWLEY RATS

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FPV 2010 20
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MASTER OF VETERINARY SCIENCE
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By

HUTHEYFA A-H AL-SALIH

Thesis Submitted to School of the Graduate Studies, Universiti Putra Malaysia, in fulfillment of the requirements for Degree of Master in Veterinary Science

December 2010
DEDICATION

To

My beloved parents, my beloved wife, my brothers, my sisters and special dedication to the new members in the family, my daughter Remas and my son Asir
Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Veterinary Science

EFFECTS OF *Morinda citrifolia* ON N-METHYL N-NITROSOUREA-INDUCED PERIPHERAL T CELL NON-HODGKIN’S LYMPHOMA IN SPRAGUE DAWLEY RATS

By

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December 2010

Chairman: Associate Professor Jasni bin Sabri, PhD

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N-methyl N-nitrosourea (MNU) is a carcinogen that had been reported to be able to cause malignant lymphoma and/or leukemia to the lympho-hematopoietic system. *Morinda citrifolia* is a known anti-tumor medicinal herb. The present study was conducted to investigate the ability of MNU to induce T cell non-Hodgkin’s lymphoma in male Sprague Dawley rats and to investigate the anti-tumor effects of *Morinda citrifolia* on affected rats.

Sixty four 8 weeks old male Sprague Dawley rats were divided into four groups of sixteen rats in each group. Groups B (MNU treated) and D (*Morinda citrifolia* and MNU treated) rats received four consecutive intraperitoneal injections of MNU at a dose of 60 mg/kg body weight, while groups A and C rats received four consecutive intraperitoneal injections of normal saline. Groups C and D rats were fed daily with a ration mixed with *Morinda citrifolia* fruit powder at a dose of 750 mg/kg body weight.
Groups A and B rats were fed with *Morinda citrifolia*-free ration. The rats were sacrificed after 20 weeks of experimental period by bleeding following xylazine and ketamine anaesthesia. Complete gross examination and weighing of the organs were conducted. Samples of the lymph nodes, spleen, liver, lung, kidneys, heart, thymus, stomach, large intestine and small intestine were collected for histopathology and immunohistochemistry. The peripheral blood was collected for haematology and blood biochemistry.

Lymph nodes enlargement was observed in five rats (32%) of group B and one rat (6%) of group D. Hepatomegaly and splenomegaly were observed in four rats (25%) of group B and one rat (6%) of group D. Enlargement of kidneys was observed in two rats (12%) of group B. Histopathology revealed lymphoma in the enlarged organs and in the lungs, kidneys and heart of groups B and D rats. The lymphoma lesions were characterized by the proliferation and/or infiltration of undifferentiated, small to medium, bizarre pleomorphic neoplastic lymphocytes with evidence of mitosis in the lymph nodes, spleen, liver, lungs, kidneys and heart of groups B and D rats. However, the thymus, stomach, large intestine and small intestine appeared normal. Groups A and C rats showed normal histology in all organs. The statistical analysis of lesion scoring results of lymphoma lesions showed significant (p<0.05) differences in the lymph nodes, spleen, liver, lungs and kidneys of group B rats compared with rats in other groups. The neoplastic lymphocytes in affected organs showed positive expression to T cell marker (CD3) and negative expression to B cell marker (CD79α) which confirmed that the lymphoma which was observed is T cell non-Hodgkin’s lymphoma.
Lymphatic leukemia was observed in groups B and D rats where severe lymphocytosis was observed in four rats (28%) of group B and one rat (6%) of group D. The RBC and HGB count results showed anemia in seven rats (50%) of group B and two rats (18%) of group D and the manually counted packed cell volume results showed anemia in four rats (28%) of group B and two rats (18%) of group D. Groups A and C rats did not show any abnormal blood parameters. The blood biochemical results showed significant (p<0.05) increased in aspartate transaminase (AST), alanine transaminase (ALT), bilirubin, urea and creatinine levels in group B rats and the uric acid levels also significantly (p<0.05) increased in group B rats and group D rats. The lactate dehydrogenase (LDH) levels decreased in group B and group D rats. Group C rats did not show any blood biochemistry changes in the serum parameter levels.

Therefore, it can be concluded that the gross pathology, histopathology, immunohistochemistry and blood biochemistry analyses revealed that the MNU can induce T cell non-Hodgkin’s lymphoma in male Sprague Dawley rats. Haematology results showed that the MNU can also induced lymphatic leukemia accompanied with anemia in the male Sprague Dawley rats.

This study also showed that the Morinda citrifolia fruit powder at a daily dose of 750 mg/kg body weight had the ability to reduce the peripheral T cell non-Hodgkin’s lymphoma, lymphatic leukemia and anemia induced by MNU in the male Sprague rats.

**Keywords:** Peripheral non-Hodgkin’s lymphoma, MNU, *Morinda citrifolia*, rats.
Abstrak daripada tesis yang dikemukakan kepada Senat Universiti Putra Malaysia untuk memenuhi ijazah Master Sains Veterinar

KESAN *Morinda citrifolia* KE ATAS TIKUS SPRAGUE DAWLEY YANG DIARUH LIMFOMA NON-HODGKIN JENIS SEL T MELALUI PEMBERIAN N-METHYL N-NITROSOUREA

Oleh

HUTHEYFA A-H AL-SALIH

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Enam puluh empat tikus jantan Sprague Dawley berumur 8 minggu telah dibahagikan kepada empat kumpulan yang mengandungi enam belas tikus bagi setiap kumpulan. Tikus dalam kumpulan B (disuntik MNU) dan D (*Morinda citrifolia* dan disuntik MNU) menerima suntikan MNU pada dos 60 mg/kg berat badan, sementara tikus dalam kumpulan A dan C menerima suntikan salin normal. Kumpulan C dan D diberi rumusan makanan yang dicampur dengan serbuk buah *Morinda citrifolia* pada dos 750 mg/kg

Pembesaran kelenjar limfa diperhati pada lima ekor tikus (31%) dalam kumpulan B dan seekor (6%) dalam kumpulan D. Pembesaran ginjal diperhati pada dua ekor tikus (12%) dalam kumpulan B. Histopatologi mendapati limfoma dalam organ yang membesar ini dan dalam paru-paru, ginjal dan jantung tikus dalam kumpulan B dan D. Lesi limfoma dikenalpasti menerusi proliferasi dan infiltrasi limfosit neoplastik tiada-pembezaan, kecil hingga besar, pleomorfik ganjil dengan mitosis dalam kelenjar limfa, organ limfa, hati, paru-paru, ginjal dan jantung tikus dalam kumpulan B dan D. Walau bagaimanapun, kelenjar timus, perut, usus besar dan kecil nampak normal. Tikus kumpulan A dan C menunjukkan histologi normal dalam kesemua organ. Analisa statistik melalui keputusan skor lesi untuk lesi limfoma menunjukkan keertian yang berbeza (p<0.05) dalam kelenjar limfa, organ limfa, hati, paru-paru dan ginjal bagi tikus dalam kumpulan B berbanding tikus dalam kumpulan lain. Limfosit neoplastik dalam organ terlibat menunjukkan kehadiran positif penanda sel T (CD3) dan negatif penanda sel B (CD79α) yang membuktikan limfoma yang diperhati ialah limfoma non-Hodgkin jenis sel T.
Leukemia limfatik yang diperhati dalam kumpulan B dan D adalah limfositosis teruk iaitu empat ekor tikus (28%) dalam kumpulan B dan seekor tikus (6%) dalam kumpulan D. Keputusan kiraan sel darah merah (RBC) dan hemoglobin (HBG) menunjukkan anemia pada tujuh ekor tikus (50%) dalam kumpulan B dan dua ekor tikus (18%) dalam kumpulan D dan keputusan kiraan manual volum sel padat (PCV) menunjukkan anemia pada empat ekor tikus (28%) dalam kumpulan B dan dua ekor tikus (18%) dan kumpulan D. Tikus dalam kumpulan A dan C tidak menunjukkan sebarang parameter darah yang abnormal. Keputusan biokimia darah menunjukkan keertian (p<0.05) peningkatan aras aspartat transaminase (AST), alanin transaminase (ALT), bilirubin, urea dan kreatinin bagi tikus dalam kumpulan B. Aras asid urik juga meningkat dengan keertian (p<0.05) bagi tikus dalam kumpulan B dan D. Aras laktat dehidrogenase (LDH) menurun bagi tikus dalam kumpulan B dan D. Tikus dalam kumpulan C tidak menunjukkan sebarang perubahan pada biokimia darah dalam serum.

Oleh itu, adalah dirumuskan analisa patologi kasar, histopatologi, immunohistologi dan biokimia darah menunjukkan bahawa MNU boleh mengaruh limfoma non-Hodgkin jenis sel T pada tikus jantan Sprague Dawley. Hematologi menunjukkan bahawa MNU juga mengaruh leukemia limfatik bersama anemia pada tikus jantan Sprague Dawley.

Kajian ini juga menunjukkan bahawa pemberian harian sebuk buah Morinda citrifolia pada dos 750 mg/kg berat badan berkeboleh mengurangkan limfoma non-Hodgkin jenis sel T, leukemia limfatik dan anemia yang diaruh oleh MNU pada tikus jantan Sprague Dawley.

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I certify that the examination committee has met on 28/12/2010 to conduct the final examination of Hutheyfa A-H Al-Salih on his Master in Veterinary Science thesis entitled “Effects of Morinda Citrifolia on N-Methyl N-Nitrosourea Induced Peripheral T Cell Non-Hodgkin’s Lymphoma in Sprague Dawley Rats” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the student be awarded the degree of Master of Veterinary Science.

Members of the Examination Committee were as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the Degree of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

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School of Graduate Studies  
Universiti Putra Malaysia

Date:
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously and is not concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

HUTHEYFA A-H AL-SALIH

Date: 28th December 2010
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4.24 Photomicrograph of leukemia in a blood smear of group D rat. Wright’s stain, 400x.
LIST OF ABBREVIATIONS

MNU                  N-methyl N-nitrosourea
mg                   Milligram
kg                   Kilogram
PTCL-u               Unspecific peripheral T cell non-Hodgkin’s lymphoma
Morinda citrifolia-ppt Morinda citrifolia in alcohol precipitate
DMBA                 7, 12 dimethylbenz (a) anthracene
mm                   Micrometer
ppm                  Part per million
MGMT                 O6-Methylguanine-DNA-Mehtyltransferase
NHL                  Non-Hodgkin’s lymphoma
NK                   Natural killer
HTLV-1               Human T leukemia virus
EBV                  Epstein barr virus
WHO                  World human organization
LDH                  Lactate dehydrogenase
ALT                  Alanine transaminase
AST                  Aspartate transaminase
PTCL-u               Peripheral T cell non-Hodgkin’s lymphoma-unspecific
SAR                  Superoxide anion free radicals
LPO                  Lipid hydroperoxide
IL                   Interleukin
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin dye</td>
</tr>
<tr>
<td>G</td>
<td>Gram</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius degree</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Round per minute</td>
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<tr>
<td>PCV</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>DAB</td>
<td>Diaminobenzidine</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>IPI</td>
<td>International prognostic index</td>
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N-methyl N-nitrosourea (MNU) is a model of carcinogenic chemicals based on sufficient evidence of carcinogenicity in experimental studies. MNU induced both benign and malignant tumors in various organs depending on the species and the routes of administration (IARC, 1978).

MNU has a broad spectrum of target organs especially the lympho-hematopoietic system. These include lymph nodes, thymus, spleen, liver, lungs, kidneys, heart, thymus, urinary bladder and intestines. Administrations of MNU at high sub-lethal doses characteristically induced early and high incidence of leukemia and/or malignant lymphomas in young rats (Uwagawa et al., 1991). Koestner et al. (1977) induced thymic lymphoma in Sprague Dawley rats using a single intragastric injection of MNU at a high dose of 350 mg/kg body weight. Thymic lymphoma had been induced in male Fischer rats using twelve intraperitoneal injections of MNU at a dose of 20 mg/kg body weight (Mizoguchi et al., 1993).

Using different doses, thymic lymphoma was induced in male Wistar rats using four consecutive intraperitoneal injections of MNU at doses of 20, 40, 60 mg/kg body weight. These studies investigated the induction of lymphoma in the thymus, lymph nodes, spleen, liver and peripheral blood only (Franchi et al., 2003). Changes in other organs of vital importance such as the lungs, kidneys and heart had not been documented. Therefore, in the present study, the metastatic potential of this neoplasm in
rats as model for human lymphoma in the vital organs such as lungs, kidneys, heart, as well as the stomach, large and small intestine was investigated. Furthermore, the confirmation and investigation of the induced lymphoma using immunohistochemistry technique which had been lacking should be ascertained.

*Morinda citrifolia* (mengkudu) had been reported to have anti-tumor activity, which has been researched widely in several animal models (Locher *et al*., 1995; Wang and Su, 2001). Using *Morinda citrifolia* fruit juice in alcohol precipitate (*Morinda citrifolia*-ppt) the life of C57 B1/6 mice implanted with lung carcinoma had been prolonged for up to 75% compared with the control group mice (Hirazumi *et al*., 1994). Many chemical compounds extracted from *Morinda citrifolia* can inhibit the activity of many important oncogenes associated with various tumors (Hiramatsu *et al*., 1993; Hiwasa and Arase, 1999).

*Morinda citrifolia* fruit juice can prevent mammary gland cancer induced by 7, 12-dimethylbenz (a) anthracene (DMBA) in female Sprague Dawley rats. DMBA treated rats developed epithelial hyperplasia (12.5%) and in situ carcinomas (25%). However, all rats treated with *Morinda citrifolia* fruit juice following induction with DMBA showed mild hyperplasia only. These results indicated that *Morinda citrifolia* fruit juice can prevent chemically induced mammary breast cancer (Wang *et al*., 2002).

The earlier studies in mice and rats (Hiramatsu *et al*., 1993; Hirazumi *et al*., 1994; Hiwasa and Arase, 1999; Wang and Su 2001; Wang *et al*., 2002) showed the anti-tumor effects of the *Morinda citrifolia* fruit juice or the effects of chemical extract from *Morinda citrifolia* on different models of tumors (Hirazumi *et al*., 1994; Wang and Su...
2001; Wang et al., 2002). However, the present study investigate the anti-tumor effects of *Morinda citrifolia* fruit in powder preparation on experimental-induced lymphoma in male Sprague Dawley rats using the N-methyl N-nitrosourea (MNU).

It is hypothesize that male Sprague Dawley rats that received N-methyl N-nitrosourea can develop lymphoma and *Morinda citrifolia* can prevent or limit the development of N-methyl N-nitrosourea-induced lymphoma.

The objectives of this study were to:

1. induce lymphoma/leukemia in male Sprague Dawley rats using N-methyl N-nitrosourea (MNU).

2. investigate the effects of *Morinda citrifolia* fruit powder on MNU-induced lymphoma in male Sprague Dawley rats.
REFERENCES


