PENGARUH EKSTRAK SAMBILOTO PER ORAL TERHADAP EKSPRESI VEGF, KI-67, APOPTOSIS DAN VOLUME TUMOR ADENOKARSINOMA MAMMA

(Studi pada Mencit C3H)

NUGRAHANINGSIH WH

PROGRAM DOKTOR ILMU KEDOKTERAN/ KESEHATAN
PASCASARJANA UNIVERSITAS DIPONEGORO
SEMARANG
2013

PENGARUH EKSTRAK SAMBILOTO PER ORAL TERHADAP EKSPRESI VEGF, KI-67, APOPTOSIS DAN VOLUME TUMOR ADENOKARSINOMA MAMMA

(Studi pada Mencit C3H)

NUGRAHANINGSIH WH
NIM G5A009004

Telah disetujui oleh:

Promotor

Prof.Dr.dr.Sarjadi,SpPAK
Tanggal..07 SEP 2013..

Ko-Promotor

Prof.Dr.dr. Hertanto Wahyu Subagio,MS,SpGK
Tanggal..12 SEP 2013.....

Prof.dr.Edi Dharmana,MSc,Ph.D,SpParK
Tanggal..12 SEP 2013.....

Program Studi Doktor Ilmu Kedokteran/Kesehatan
Pascasarjana Universitas Diponegoro Semarang

Ketua

Prof.Dr.dr. Hardhono Susanto, PAK(K)
Tanggal..25 SEP 2013.....

PERNYATAAN ORISINALITAS

Yang bertanda tangan di bawah ini:

Nama : Nugrahaningsih WH

NIM : G5A009004

Mahasiswa : Program Doktor Ilmu Kedokteran/Kesehatan

Pascasarjana Universitas Diponegoro

Dengan ini menyatakan bahwa:

1) Disertasi yang berjudul "Pengaruh Ekstrak Sambiloto Per Oral Terhadap Ekspresi VEGF, Ki-67, Apoptosis dan Volume Tumor Adenokarsinoma Mamma (Studi pada Mencit C3H)" adalah karya ilmiah asli dan belum pernah diajukan untuk mendapatkan gelar akademik (doktor) di perguruan tinggi manapun
2) Disertasi ini adalah murni ide, rumusan dan hasil penelitian saya, serta dilakukan tanpa bantuan orang lain, kecuali tim promotor dan narasumber
3) Disertasi ini tidak terdapat karya atau pendapat yang telah ditulis atau dipublikasikan orang lain, kecuali secara tertulis dicantumkan sebagai acuan dalam naskah dengan disebutkan nama pengarang dan judul aslinya serta dicantumkan dalam daftar pustaka
4) Pernyataan ini dibuat dengan sesungguhnya dan apabila di kemudian hari terdapat penyimpangan dan ketidakbenaran dalam pernyataan ini, saya bersedia menerima sanksi akademik berupa pencabutan gelar yang telah saya peroleh, dan sanksi lain sesuai dengan norma yang berlaku di Universitas Diponegoro.

Semarang, Agustus 2013

Yang membuat pernyataan

Nugrahaningsih WH

ABSTRAK

Latar Belakang: Beberapa penelitian secara *in vitro* menunjukkan pengaruh ekstrak sambiloto (*Andrographis paniculata*) terhadap apoptosis dan kadar VEGF serum beberapa *sel line* kanker. Adanya pengaruh lingkungan mikro terhadap pertumbuhan jaringan kanker memungkinkan hasil yang berbeda antara penelitian secara *in vitro* dan *in vivo*. Penelitian bertujuan membuktikan pengaruh pemberian ekstrak sambiloto per oral terhadap ekspresi VEGF, Ki67, apoptosis dan volume tumor adenokarsinoma mamma mencit C3H.

Metode Penelitian: Penelitian eksperimental *post test randomized control group design* dilakukan pada 24 ekor mencit C3H yang telah ditransplantasi adenokarsinoma mamma. Sampel dibagi dalam 4 kelompok yang mendapatkan ekstrak sambiloto per oral sebanyak 0 (kontrol), 5, 10 dan 15 mg/hari selama 14 hari. Volume tumor diukur dengan kaliper digital. Ekspresi VEGF dan Ki-67 diperiksa dengan imunohistokimia. Apoptosis diperiksa dengan TUNEL.

Hasil: Pemberian peroral ekstrak sambiloto 5, 10 dan 15 mg/hari menurunkan ekspresi VEGF ($p=0,000$, $r = -0,953$), ekspresi Ki-67 ($p=0,000$, $r = -0,938$) serta meningkatkan apoptosis ($p=0,000$, $r = 0,974$), tetapi belum dapat menurunkan volume tumor. Adanya korelasi antara ekspresi VEGF dan Ki-67 ($r =0,920$), ekspresi VEGF dengan apoptosis ($r = -0,957$) dan antara Ki-67 dengan apoptosis ($r = -0,931$).

Simpulan: Pemberian ekstrak sambiloto peroral mempengaruhi angiogenesis, proliferasi dan apoptosis adenokarsinoma mamma dengan indikator penurunan ekspresi VEGF, Ki-67 dan peningkatan apoptosis. Pertumbuhan adenokarsinoma mamma dipengaruhi oleh banyak faktor. Ekspresi VEGF, ekspresi Ki-67 dan indeks apoptosis merupakan bagian kecil dari indikator pertumbuhan kanker yang tidak linier dengan ukuran makro adenokarsinoma mamma.

Kata kunci: *Andrographis paniculata, apoptosis, ekspresi VEGF, ekspresi Ki-67*

ABSTRACT

Background: Several *in vitro* studies showed the influence of sambiloto extract (*Andrographis paniculata*) on apoptosis and VEGF serum levels of cancer cell lines. The influence of the microenvironment on the growth of cancer allows different results between *in vitro* and *in vivo* experiment. The aim of this research was to prove the effect of oral administration of sambiloto extract on VEGF expression, Ki67 expression, apoptosis and tumor volume of adenocarcinoma mammae C3H mice.

Methods: The post-test randomized control group design experiment performed on 24 C3H mice that had been transplanted adenocarcinoma mammae. The samples were divided into 4 groups, than were given sambiloto extract orally as 5, 10 and 15 mg/day along 14 days. The control group were given aquadest. Tumor volumes were measured by digital calipers. Expression of VEGF and Ki-67 were examined by immunohistochemistry staining. Apoptosis were examined by TUNEL method.

Results: Oral administration of sambiloto extract as dose 5, 10 dan 15 mg/day decreased both VEGF expression ($p=0,000$, $r = - 0,953$) and Ki-67 expression ($p=0,000$, $r = - 0,938$), and increased apoptotic index ($p=0,000$, $r = 0,974$). The strong correlation were found between VEGF and Ki-67 expression ($r =0,920$), VEGF expression and apoptotic index ($r = - 0,957$), Ki-67 expression and apoptotic index ($r = - 0,931$).

Conclusion: Oral administration of sambiloto influenced angiogenesis, cell proliferation and apoptosis of adenocarcinoma mammae, by the VEGF expression, Ki-67 expression and apoptotic index as indicators. Many factors influenced tumor growth. VEGF expression, Ki-67 expression and apoptotic index, a few of tumor growth indicator which not linear from macroscopic of adenocarcinoma mammae.

Key word: *Andrographis paniculata; apoptosis; VEGF expression; Ki-67 expression*

SUMMARY

A. BACKGROUND

Mammary cancer was a common malignancy found in women worldwide and the secondary cancer that cause death. The standard of therapy for mammary cancer according to WHO guidelines was by surgery, radiotherapy, sitostatika and hormonal. The result of that therapy had not given satisfactory due to the high number of mortality. Many efforts and research were performed to obtain optiinal outcomes by inhibiting cell proliferation, increasing apoptosis, and cut off the supply of nutrition of tumor.

Sambiloto, one of the traditional herbs was used widely in Asian countries. Andrographolide isolated from sambiloto has anticancer activity through the mechanism of apoptosis on HeLa cancer cells and induces apoptosis TD-47 cell line of Human Breast Cancer. The treatment of aquadest extracts on adenocarcinoma mammae cells cultured from C3H mice increased apoptotic cells. In vitro research of several types of cancer cell line which were treated sambiloto extract showed a declining trend in the levels of VEGF in prostate cancer cell line PC-3.

The study using extracts sambiloto conducted in vitro indicate its role against cancer cells. The influence of microenvironment on cancer growth in associated with therapy allowing different results between in vitro and in vivo study. In vivo study was needed to improve the anticancer effect of sambiloto extract.

B. METHODS

The research were performed by *Randomized post test control group design*.. A total of 24 C3H mice that had transplated adenocarcinoma mammae were randomly divided into 4 groups of 6. The samples were given sambiloto extract, its doses 5, 10 and 15 mg per head per day. The control group were not given sambiloto extract. Sambiloto extract were given orally using a gavage sonde. Sambiloto extract was given in the form of liquid by aquadestilata. Sambiloto extract was given a time daily for 14 days. Tumor volume measurements performed on days 1, 4, 8, 12 and 15. On day 15 mice were terminated by ether inhalation. Cancerous tissue were prepared into paraffin blocks for immunohistochemistry examination

of TUNEL, the expression of Ki-67 and VEGF. During maintenance, mice get food and water ad libitum.

C. RESULTS

Compared control group, VEGF expression of mice which administered sambiloto were lower. VEGF expression were decreased after oral administration of sambiloto extract as dose 5 mg/day (p=0,002), 10 mg/day (p=0,000) and 15 mg/day (p=0,000). The Pearson correlation test showed a strong correlation between dose and VEGF expression (r = - 0,953).

Expression of Ki-67 were decrease after oral administration of sambiloto extract 5 mg/day (p=0,000), 10 mg/day (p=0,000) and 15 mg/day (p=0,000). There were no different effect between 10 and 15 mg/day of sambiloto administration (p=0,110). The Pearson correlation test showed a strong correlation between dose and Ki-67 expression (r = - 0,938). The strong correlation also found between VEGF expression and Ki-67 expression (r =0,920).

Apoptotic index were increase after oral administration of sambiloto extract as dose 5 mg/day (p=0,000), 10 mg/day (p=0,000) and 15 mg/day (p=0,000). There are the strong correlation between dose and apoptotic index (r = 0,974), VEGF expression and apoptotic index (r = - 0,957), Ki-67 expression and apoptotic index (r = - 0,931).

Administration of sambiloto extract as doses 5, 10 and 15 mg have not been able to decrease tumor volume (p = 0.367). Decreasing of the tumor volume fold (p=0,049) showed the inhibitory of cancer growth. Ki-67 expression contributed on decreasing of the tumor volume fold (p=0,013, r=0,499), and also the dose of extract (p=0, 042, r= - 0,419).

D. DISCUSSION

Microenvironment have important role on tumor growth. The microenvironment included immun response, oxygen level, supply of nutrition, hormones and etc. These factors implicated on the different result of in vitro and in vivo study, although the dose of bioactive were proportional.

Oral treatment of sambiloto extract caused it through of absorption, distribution, metabolism and excretion process in the body of mice. The process was very influential on the effects on target cells. There was coformational structure of sambiloto extract during the process. The change bioactive structure implicated a different result of the same molecule.

The result of study showed the lower of VEGF expression on experiment mice than the control. The decreasing of VEGF expressionn suggested the effect of sambiloto extract on angiogenesis when administered oral, direct on culture or intraperitoneal injection.

The rapid of tumor growth caused excalation of nutrition and oxygen necessity. Hipoxia stimulated releasing of proangiogenic factor, HIF-1. The complex protein of HIF-1 binded to sequence og VEGF-A gene, erythropoetin gene (EPO) and other gene associated to glycolitic enzyme and glucosa transport. Glycolitic enzyme and glucosa transport involved on biological aspect of cancer which as cell immortality, genetic unstable, maintenance of stem cell, energy and glucosa metabolism, vascularisation, invasiveness, metastase and resistance of drug therapy.

Administration of sambiloto extract decreased VEGF expression by way of inhibitory VEGF ligand and its receptor. Sambiloto extract sensitisized TRAIL (TNF-Related Apoptosis –inducing ligand). TRAIL affected to VEGF and apoptosis. TRAIL activated PI3K/Akt and ERK pathway. Activation of PI3K/Akt and ERK pathway was a down-regulation of HIF-1 and VEGF transcription. PI3K/Akt signaling pathway influenced cell survival and apoptosis. PI3K act on target serine-threonin Akt which promote cell survival. Several substrate Akt protein involved regulation of apoptosis. Many substrate Akt role as proapoptosis inaktivated by phosphorilation, i,e FOXO and Bad. Phosphorilation of Akt caaused FOXO stay in cytosol and the proapoptotic gene were not expressed. Bad inhibited antiapoptotic family member og Bcl-2, that was Bcl-xl. Phosphorilation of Akt lead translocated Bad to cytosol and neutralized apopyotic activity resulted cell survival.

The mechanism of angiogenesis altered was inhibitory effect of NO. NO were derivated from L-arginine and catalisated by *nitric oxide synthetase* (NOS). NOS catalisated NO became citruline and free NO. Endothelial cell expressed endothelial NOS (eNOS) responsible of regulation NO produce. Increasing of eNOS activity have a positive correlation with vascular density and tumor growth.

VEGF expression associated with formation of new vessel purposed energy supply needed to metabolism. In stark contrast to physiological neovascular response, the cancer is often characterized by chaotic growth, patterning and dysfunction. During tumorigenesis,

endothelial cell proliferation rapidly increased, with turnover time decreasing from approximately a thousand days in quiescent vessel beds to 50-60 hours. In addition to assuming a chaotic structure, the vessel in cancer form tortuous and variated in size and morphological.

Host tissue an important factor influenced patterning of growth vessel by providing local cues to control branching morphogenesis, the copatterning of vessel and nerves, positioning and connectivity of the arterio-venous compartement. Elaboration of vessel pattern on tumor was most likely not recapitulated during tumorigenesis, were caused by disorganized, heterogeneous and chaotical expanding tumor mass. Lack of genetic was another factor associated cell transformation and malignancy induced instability between stimulator and inhibitor of angiogenesis.

.VEGF expression were lower in experimental mice than control group. It means that active cell on G_1, S, G_2 and M phase were decreased. G_2/M checkpoint prevent the DNA damage cell enter to M phase. G1/S checkpoint involved p21 activity. When p21 binded CDK1 complex, cell can not follow to next phase. p27 binded another CDK which in complex cyclin E/CDK2 and cyclin A/CDK2 lead cell cycle arrest in G1 phase.

The checkpoint concept suggested the mechanism of cell cycle arrest when the stressor act caused DNA damage. The cycle cell would stop unrandomly specificity to repair the damage before enter to the next step of cycle cell. There are several checkpoint along during cyce cell, with resulted delay or stopped of cycle on spesific point as respon to DNA damage. The activation of protein involved cell cycle arrest lead expression of DNA repair gene and apoptotic gene. DNA repair pathways functionaly when DNA damage induced cell cycle arrest. Apoptosis could occur independently on cell-cycle arrest machinery when the dNA repair failed.

Decreasing of cell accumulation that in active phases of proliferation forward to apoptosis or enter to cell cycle. The cell forward to apoptosis when DNA repair mechanism failed. In opposite, cell enter into cell cycle when DNA repair mechanism successfull and cell be normaly. Both apoptosis and successfully of DNA repair mechanism suggested the change of cell behaviour nearly the normal.

There was two pathway that trigger apoptosis, the extrinsic and intrinsic apoptotic pathway. Sambiloto extract stimulated apoptotic both extrinsic and intrinsic pathway.

Andrographolide initiated apoptotic intrinsic pathway via p53 regulation. Internal signal caused disassociated of Apaf-1 from Bcl-2. The Apaf-1 were accompanied to cytochrom-c, which together with caspase 9 and ATP formed apoptososom. The apoptosom complex activated pro-caspase 3 lead to active caspase-3. Caspase 3 was an executioner of cascade caspase. Andrographolide from sambiloto extract activated caspase 8 and initiated extrinsic apoptotic pathway, and than activated caspase 9 and caspase 3, as a executor caspase. The Extrinsic signal of apoptosis were formed by complementary binding of Fas and TNF, which role as *death activator*. The signal from *death activator* leading to cytoplasm and activated caspase 8. The active caspase 8 initiated cascade caspase and triggered apoptotic process. Another way, caspase 8 activated Bid and initiated apoptotis pathway via enhanced Bax and Bak activity

Cell population of tissue were determined by the balancing of tumor expansion and tumor involution. Disturbing the balance of these process result in disease, if cell losses exeed renewal this results in involution, whereas the converse results in tissue expansion. In this research, cell proliferation of experimental group that received sambiloto extract were lower than control group. In opposite, apoptosis index were increased. Although the cell proliferation was decreased and apoptosis index was increased, the tumor volume had not yet reduced. Actualy, tumor massa contain not only cancer cell, but also stromal tissue, blood vessel, immun cell, apoptotic and necrotic cell and etc.

The high expression of VEGF might an important key contributed on tumor growth. Nutrition and oxygen demand were elevated as caused of rapid proliferation of cancer cell. VEGF stimulated endothelial cell to form new blood vessels provided burst of nutrition and oxygen demand. Fullfilling of nutrition oxygen demand lead cell proliferation freely. Availability of nutrition and oxygen also inhibit apoptotis. Apoptosis index in this research already low.

Microenvironment cell included extracelullar liquid and stromal tissue were a environment provided nutrition, mineral, hormones and others that need to cell grow. Microenvironment played an important role on tumor growth, provided a media of cell signaling and networking. Stromal cell included fibroblast, perisit, mesenchimal stem cell and hematopoeticc cell. Stromal cells help cancer growth by various mechanism, e.g influenced tumor vasculature dan released VEGF.

Drug and other agent were given orally would be process through the absorbtion, distribution , metabolism and excretion. The process was influenced by many factors such as the physiology of gastrointestinal, absorbtion mechanism such as active transport, carrier protein (transporter) expression and individual pathological condition. Carrier protein excreted in several organs such as intestinal, liver, kidneys and brain could affect ADME process. Poliglycoprotein (PgP) was a transporter that serves as a repellent chemical compounds or drugs that came out of the organ, included cancer

Immune system influenced tumor growth caused their ability to kill cancer cell, that as effector mechanism to cancer. Cancer cell could escape from immun attack by varius mechanism and grow. The small number of cell were tranplanted could escape from immun attack via sneaking through mechanism. The small number of cell were not adequate to stimulated immun system. The other tumor escape mechanism was antigen masking, surface antigen of cancer was hidden from immun attack in way glicocalic cover.

Many factor contributed to tumor volume suggested the role of overall part included gene, cell, tissue, organ and system organ. All of component involved in vivo study not autonomous, but composed a network in function.

F. CONCLUSION AND RECOMMENDATIONS

In vivo study oral administration of sambiloto extract influenced angiogenesis, proliferation and apoptosis of adenocarcinoma mammae C3H mice. The decreasing of VEGF and Ki-67 expression and increasing of apoptotic index were indicators of sambiloto effects on angiogenesis, proliferation and apoptosis. The VEGF and Ki-67 expression have not yet reach the its expression on non neoplastic tissue. The oral administration of sambiloto extract could not decreasing of tumor volume.

Adenocarcinoma mammae tumor growth was a complex process. Many factor affected the tumor growth. VEGF expession, Ki-67 expression and apoptotic index was a few part of tumor growth indicators. The change of VEGF expession, Ki-67 expression and apoptotic index was not linear with tumor volume.

This study was not perfect. Beside of the limitations of research, there were many other variables that can not be examined in this research. Other study were required to support the use of sambiloto extract as anticancer such as the percentace of cancer cell, stromal tissue, and

necrotic cell which formed tumor mass. The experimental using combination between sambiloto extract and other standardized cancer therapy were needed.

DAFTAR ISI