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EFFECT OF GRAPE SEED EXTRACT (Vitis vinifera, sp) ON HISTOPATHOLOGY APPEARANCE, cMyc AND Bcl-2 GENE EXPRESSION OF AOM-DSS INDUCED MICE

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Introduction: The use of Grape Seed Extract (GSE) as a dietary supplement to prevent cancer in Indonesia is increasing. The extract content such as resveratrol, quercetin, epigalokatecin and proanthocyanidin, are reported to increase apoptosis and prevents cancer cell proliferation which is shown by the expression level of cMyc and Bcl-2 gene. Azoxymethane (AOM) and Dextran sulfate sodium (DSS) are two chemical compounds used to induce cancer in mice. Methods: Fifteen female mice were divided into 3 groups, whose treatment was sequentially as follows: I. Induced by AOM-DSS; II. Induced by AOM-DSS and EBA; and III. Neither induced by AOM-DSS nor given EBA. Histopathology examination of the colons was presented in descriptive report. Analysis of cMyc and Bcl-2 gene was performed; the gene expression level was determined using the Livak method and statistically analyzed using the Mann-Whitney test. Result: Dysplasia were shown in group I, mild dysplasia over the repair tissue in group II, and healthy tissue in group III. Using the Livak method, cMyc and Bcl-2 gene expression of group II are 1.4 times stronger and 1.9 times stronger than group I. But statistically both of the result are non-significant (cMyc, P = 0.251; Bcl-2, P = 0.754). Discussion: Grape seed extract has no effect on the decrease in cMyc and Bcl-2 gene expression in AOM-DSS induced mice. The extract dose used has anti-inflammatory effect, which was shown on mice colon tissue histopathology but no anti cancer effect. To get the anticancer effect, it takes GSE with higher doses. In further research, it is necessary to use multiple doses of the GSE in order to know the effective dose to prevent the occurrence of colon cancer in mice induced AOM-DSS.

Keywords: Azoxymethane-Dextran Sulphate Sodium, Bcl-2, c-Myc, Colon Cancer, Grape Seed Extract

1. INTRODUCTION

The use of dietary herbal supplement is increasing nowadays. Many people seek for the beneficial effect of the dietary supplement such as, immune system boosting, or even anti-cancer effect. One of the dietary supplements broadly used is grape. Grapes is known as fruit that contain so many active compound such as resveratrol, quercetin, epigalokatecin, proanthocyanidin, which has been proven to act in multiple target in reducing the in vitro cancer cell proliferation---(Parry J, et al. 2006). Grape seed extract gives the easier preparation and more concentrated dosage to be given to the user.

Some cancer research has been conducted in vitro using cell line and in vivo using the animal model. One of the animal model was using the wild type mice induced by chemical
compound such as *Azoxymethane* (AOM), a synthetic carcinogen that induced gene mutation, and *Dextran sulphate sodium* (DSS), a chronic inflammation agent. Histologically, the effect of AOM and DSS administration to mice are dysplasia, polyp, adenoma, and adenocarcinoma---(Tanaka, 2012; Wicaksono, 2013).

Cancer development involves the cell cycle and apoptosis process, which is regulated by genes. Two of many genes that regulate cell cycle and apoptosis process are cMyc gene and Bcl-2 gene. cMyc is a transcription factor coding gene, where its mutation frequently found in cervical cancer, breast cancer, lung cancer, and many other cancer including colon cancer---(Albinh, et al. 2010). Bcl-2 is an anti-apoptosis gene that presents frequently in cancer. The increase in Bcl-2 expression will hamper the apoptosis process of the cell, and if there are alteration of cell proliferation, it can lead to development of cancer---(Rajguru, 2012)

Previous research shows that supplementation of grape seed extract can hamper the proliferation of colon cancer cell line DU145 and HT29 that are transplanted in nude mice---(Velmurugan, 2010). GSE has a strong inhibiting effect to HCT-8 and Caco-2 colon cancer cell line proliferation, and also increase in apoptosis of those cells, observed from the apoptosis protein expression---(Dinicola, 2012). This research were conducted to understand the effect of grape seed extract to the expression level of cMyc and Bcl-2 genes as a marker of cell proliferation and apoptosis regulation, also histopathology appearance of the treated mice will be observed.

2. MATERIAL AND METHODS

2.1. Animal Model

Using the Mead Equation, 12 Balb/C female mice (*Mus musculus, sp*), 18-22 gram, 6-8 week of age, were obtained from the Animal Laboratory, Department of Pharmacology and Therapy, Faculty of Medicine, Universitas Padjadjaran. The mice were housed in cages and kept under at a room temperature of 20–22°C, with 12 h light and 12 h dark cycle. They were allowed to acclimatize for 1 week before the experiments were started. Mice were divided into 3 groups. Group I were induced by AOM and DSS---(Tanaka, 2012); Group II were induced by AOM and DSS---(Tanaka, 2012) and given the grape seed extract; the Group III were neither induced by AOM and DSS nor given the grape seed extract.

2.2. Induction with AOM and DSS, and GSE Suplementation

*Azoxymethane* (AOM), is an alkylating agent that alter the DNA and initiate cancer, especially for the development of colon cancer mice model. This compound was injected intraperitoneally with single dose 10 mg/kgBW/mice. This procedure was done on 7th day after adaptation. *Dextran Sodium Sulfate* (DSS), is an inflammatory agent that can induce colitis in rodents. The process was aimed to make the chronic colitis and develop into colon cancer. The DSS was administered orally ad libitum, with 2% concentration (w/v) at day 14 to day 20. Grape Seed Extract (GSE) was administered orally starting from day 21 until day 76 to group II, the mice was given 0,5 ml, equal to 5,6 mg of GSE. The dose was 280mg/kgBW, converted from rat dose of Cheah’s prior research 200mg/kgBW using the conversion table. (Cheah, 2012; Lurence & Bacharach, 1964). The GSE was dissolved in CMC
2.3. Collecting Tissue Sample for mRNA Extraction and Histopathology Examination

At the day 77th, all of the mice were sacrificed using 0.04% (v/v) of Ketamine and cervical dislocation. The colon mucosa morphology was observed, and small part of tissue were collected. About 0.5x0.5 cm of colon were preserved using RNALater for mRNA extraction and other small part were collected in 10% formaldehyde for histopathology examination. Extraction of mRNA was using MagNA Pure®. The tissues were preserved in 10% formaldehyde then sent to laboratory of Department of Anatomy Pathology Hasan Sadikin General Hospital to be processed for histopathology examination by Pathologist.

2.4. RT-PCR for C-Myc and Bcl-2 RNA Analysis

Reverse Transcriptase PCR procedure was using the KAPA® RT-PCR Kit from 1st BASE. For the c-Myc gene, the primer pair were F: 5’-TACCCTCTCAACGACAGCAG-3’ and R: 5’-TCTTGACATTCTCTCGGTG-3’. For the Bcl-2 gene, the primer pair were F: 5’-GTGGAGGAGCTCTCAGGGA-3’ and R: 5’-AGGCACCAGGGTGATGCAA-3’ (Field 2001; Gradilone 2003).

3. RESULT

3.1. C-Myc Gene Expression

C-Myc gene expression of group I (induced by AOM-DSS) were significantly higher than group III (control) (Table.3.1), but if the group I (induced by AOM-DSS) were compared with group II (induced+GSE treatment), the c-Myc gene expression were higher for group II than for group I. (Table.3.1).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (min-max)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>9,95 (8,6 – 11,57)</td>
<td>0,009**</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>12,99 (11,64 – 16,53)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>8,94 (8,5 – 10,5)</td>
<td>0,251</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>9,95 (8,6 – 11,57)</td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney Test; **significant if p<0,05

Using the Livak Method to quantify the power of gene amplification, it was concluded that c-Myc gene expression of group I are 8,2 times higher than group III. With the same method, it was concluded that c-Myc gene expression of group II were 1,4 times higher than group I.

3.2. Bcl-2 Gene Expression

Bcl-2 gene expression in group I (induced by AOM-DSS) were significantly higher than group III (control) (Table.3.2), but if the group I (induced by AOM-DSS) were compared with group II (induced+GSE treatment), the Bcl-2 gene expression were insignificantly different. (Table.3.2). Using Livak Method showed that Bcl-2 gene expression of group I are 8,2 times higher than group II. The power of Bcl-2 gene expression are 1,9 times higher of group II than in group I.
Table 3.2: Comparison of Ct (Cycle threshold) of Bcl-2 gene between group tested

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (min-max)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>14.84 (12.41 – 16.89)</td>
<td>0.047**</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>18.53 (15.37 – 19.86)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>18.86 (15.89 – 19.86)</td>
<td>0.754</td>
</tr>
<tr>
<td>I</td>
<td>5</td>
<td>14.84 (12.31 – 16.89)</td>
<td></td>
</tr>
</tbody>
</table>

*Mann-Whitney Test; **significant if p<0.05

3.3. Histopathology Description

The histopathology appearances for the groups involve in this research were explained in descriptive, the resume can be seen in Table 3.3.

Group III, as a normal mice control showed assumedly normal in histopathology appearance. Generally in this group, the colonic mucosal lining showed the non-inflammatory mucosa; loose stromal, and non-hyperplastic lymph nodes. Epithelial lining neatly arranged with dark nucleus at the basal zone of the cells. No Polyp appeared in all sample. Riddel criteria showed no epithelial destruction, no abnormality in crypts, and goblet cell, no pleomorphic, and normal cell polarity. There were also no inflammations, only 1 sample showed erosions with PMN as acute inflammation indication.

Table 3.3: Resume of Histopathology description of the mice colonic tissue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Epithelial destruction</td>
<td>3 out of 5</td>
<td>3 low grade erosion</td>
</tr>
<tr>
<td>Cript</td>
<td>Decreased &amp; disappeared</td>
<td>Mostly normal</td>
</tr>
<tr>
<td>Goblet cell</td>
<td>Decreased &amp; disappeared</td>
<td>2 decreased</td>
</tr>
<tr>
<td>Pleomorphic</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Cell Polarity</td>
<td>altered</td>
<td>2 altered</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>Adenoma</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Other</td>
<td>Polyp (+)</td>
<td>Polyp (-)</td>
</tr>
<tr>
<td></td>
<td>Hyperplasia Lymph (+)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Microscopic appearance of mice colon in group I.
(a)10x obj. magnification shows the polyp development at the mice colon mucosa (yellow arrow); (b)40x obj. magnification shows hyperplastic mucosal lining (yellow arrow: hyperplastic mucosa)

Group I that was induced by AOM and DSS without GSE supplementation showed the inflammation process with the hyperplasia of colon lymph node, dysplasia in epithelial cell marked with the clear nucleus and prominent nucleolus. There were also polyp appearances without dysplasia of the epithelial cell, so that could be non-adenoma. Based on Riddel criteria there were epithelial destruction erosions form in 3 of 5 sample, colonic tissue crypts decrease and disappeared in some of sample, goblet cell decreased and disappeared in some of sample, all of group 1 sample showed the pleomorphic appearance, cell polarity was shown altered in almost all sample---(Riddel, et al. 1983). Picture can be seen in Figure 1.

Group II (induced by AOM-DSS + GSE supplementation) showed the repaired epithelial lining. Two of five sample showed no epithelial destruction, and the other three showed low grade erosions with repaired epithelial lining. Colon mucous crypts mostly showed normal appearance compare to group I. It was showed a shortening in crypts and some stromal showed edematous appearance. Goblet cell showed variation, 2 out of 5 sample showed decrease in number. No cellular pleomorphism showed in this group. Dark nucleus and no prominent nucleolus was observed. Cell polarity were altered in two from five sample observed. Inflammatory cell showed in group II colonic mucous, limphocyte bunch, mucous architecture poorly arrange. Group II histopathologic appearance showed that there are inflammation apoptosis and necrosis.

![Figure 2: Microscopic appearance of mice colon in group II](image1)

(a)10x obj. magnification shows the darker and compact mucosal lining due to inflamatory cells infiltration; (b)altered mucosal lining architecture due to nectosis and apoptosis

4. DISCUSSION

The procedure for mice induction used the protocol from Tanaka---(Tanaka 2012). The result in macroscopic observation and microscopic examination was different, the previous research observe the 8th, 9th and 14th week sacrificed mice has developed the adenocarcinoma. In this research, mice were sacrificed at the 9th week of the study, but there’s no marked abnormality in the colon mucosal lining. Expression level of cMyc and Bcl-2 genes were examined, and shown that there was significant increase of expression level of those genes. The result, could be influence by many things such as the environment, nutrition, and the mice factors, so that the alteration was in the genomic level and didn’t appear at the phenotype level---(Tanaka 2012).
In the GSE supplemented group (group II), the result showed the increase of the cMyc expression 1.4 times than the control, and it showed the contradiction between the hypothesis and the theory proposed. The expression of cMyc gene are increasing in cell proliferation, it was shown in the AOM-DSS induced mice, which is 8.2 times stronger compare to the non-induced mice. The contradiction can be due to the double standard of herbal usage, they neither can be an anti-oxidant, nor pro-oxidant. Most mechanism of chemotherapy drugs is to “kill” the cancer cell through the ROS induced apoptosis. However, the usage of herbal drugs or supplement, at certain concentration can eliminate the ROS, so it can inhibit the apoptosis process, or even cause the resistance of next chemotherapy---(Watson, 2013). The GSE has been analyzed to have the high polyphenol concentration such as Resveratrol, Epigallokatekin, Quercetin and Proanthocyanidine, which is having the antioxidant (ROS eliminating) effect.---(Shi, 2003). Based on this fact, we can conclude that low dose of polyphenol cannot solve the cancer problem, yet it can promote the tumor level due to the lack of ROS as main factor for damaged cell elimination. The same pattern also been shown in the Bcl-2 gene expression, which is has the role as an anti-apoptosis gene. In group II, they also increase 1.9 times compare to the group I, showed that the GSE in given concentration can lead to the inhibition in apoptosis---(Chen, 2009; Joshi, 2001).

Histopathology appearance was examined using the Riddel criteria and based on the review of two Pathologists. All sample from group III, which are not induced by AOM and DSS, are in the normal appearance. One sample showed an acute inflammation process (seen by PMN infiltration) is assumed not because of the tumor or cancer, because all of the risk factor for tumor/cancer has been eliminated. Distinct differences has been showed in the group I, which is induced by AOM and DSS---(Tanaka 2012). Microscopically, it was seen to be low and medium grade of epithelial destruction that can be due to the effect of DSS. Decreased or disappeared on colonic crypts indicate the destruction of colonic mucosal lining, as well as the decrease of goblet cells. It is also showed the alteration of cell pleomorphic and cell polarity, which are indicate the poor differentiations and cell regeneration---(Kumar 2013). The tumorigenesis process involved the induction and promotion process. Promotion process is happened due to the DNA alteration by AOM metabolites named methylazoxymethane. DSS used in this research take a role as a colonic inflammation agent and had a cytokine-inducing role in the colonocyte cytoplasm, later causing chronic inflammation that can lead to tumorigenesis ---(Laroui, 2012; Meira, et.al. 2008). The synergistic effect of DNA damage (in the proliferation site) with the anti-apoptosis function alteration (regulate by Bel-2) can lead to uncontrolled proliferation and develop a tumor. In this case, the GSE that supplemented to the induced mice can eliminate the ROS produced as the effect of DSS administration, so that the inflammation process from the DSS can be inhibited. It was seen at the group II microscopic appearance: low-grade erosion of the mucosal lining and decreased goblet cells as the result of inflammation and epithelial destruction due to DSS administration. There are also alterations in cell polarity, which can be due to the repairing process of the colon mucosa. However there are also the normal appearance of mucosal crypts, as well as no dysplasia and adenoma.

GSE is potential to have the good effect due to its chemical compound content, can prevent the development of cancer when consumed regularly. However, the information about the curing effect of the compound like resveratrol, epigallokatekin, quercetin and Proanthocyanidine is limited, and it might need high concentration of the compound to eliminate the cancer (if the cancer is already present), which is not applicable if using the regular consumption of the fruit.
5. CONCLUSION

Administration of AOM-DSS to the mice using Tanaka method significantly alter the genomic properties of mice colon mucous cell, proven by the increase of cMyc and Bcl-2 gene expression. But, using the 280mg/ml concentration of extract didn’t give the significant effect to the cMyc and Bcl-2 gene expression although there are differences of those genes expression level using Livak Method. Eventough, Grape seed extract supplementation to AOM-DSS induced mice showed repair effect on microscopic structure of the destructed colon mucous. This research need to develop again to get the optimal dose of Grape Seed Extract to get the better result of genomic alteration, especially cMyc and Bcl-2.

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