Pilot Study for *Azoxymethane-induced Colon Cancer* in *Male Wistar Rats*

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Abstract

Introduction : The use of animal models could significantly further the elucidation of Colorectal Cancer (CRC) molecular pathogenesis and help in the discovery of preventive and therapeutic agents for the disease. Dimethylhydrazine (DMH) is a widely used chemical agent for Carcinogen induced CRC model. As agent, DMH is however becoming less readily available; hence in this Pilot Study we use Azoxymethane (AOM), a DMH metabolite as an alternative agent to induce CRC in male Wistar rats.

Methods: Forty two male Wistar rats at six weeks of age were randomly assigned into negative control groups and groups receiving two AOM injections subcutaneously (SC) within one week interval at 15 mg/kg body weight (BW) and 20 mg/kg BW respectively. Rats were sacrificed 8, 16 and 24 weeks post- AOM administration. Aberrant crypt foci (ACF) were analyzed. Tumor foci were characterized by gross examination and histopathological characteristics.

Results: All rats in the AOM groups developed tumors in the colonic mucosa. Formation of ACF was detected starting from 8 weeks post-AOM injection. The highest number of ACF with multiple crypts was observed at 16 weeks post-AOM administration. The total number of ACF did not vary between the two AOM doses. Mild, moderate and severe dysplastic cells were observed in colonic mucosa starting 8 weeks post-AOM injection. There was no statistically significant difference between number of severe dysplastic cells between the two AOM doses.

Conclusion: Administration of AOM 15 mg/kg BW SC is able to induce CRC in male Wistar rats. Higher dose is not necessary since it does not result in higher tumor incidence. This cancer model may be utilized to study chemopreventive effect of various agents in the future.

Introduction

Colorectal cancer is the second leading cause of cancer morbidity and mortality worldwide. Almost half of the world's population will develop at least one benign adenomatous colonic polyp in their lifetime, with less than 3% of those cases going on to develop colorectal cancer [1]. Almost 55% of the cases occur in developed countries. In Indonesia colorectal cancer is an emerging public disease health problem and currently among the three most prevalent types of cancer [2].

Research on colon cancer has benefited from the use of *in vitro* cell culture, and biopsy specimens. However, these methods have their own limitations. Colon cancer cell lines are able to grow *in vitro* without common micro environments such as interaction with matrix and stromal cell signal. In an *in vivo* setting however colon cancer cells rely on these factors for tissue homeostasis. Additionally, biopsy specimens taken from an individual may come from tumor which has developed over a long period and acquired complex mutational changes.
Therefore, their use may limit research on tumor initiation or promotion in which the genetic and environmental diversity should be controlled. Controlled *in vivo* studies in animal models are therefore considered as

important means to understand the molecular mechanisms of colorectal carcinogenesis for development of potential preventative and therapeutic strategies using a complex physiology of the colon [3, 4].

In order to maintain the translational potential of animal models for colon cancer, some characteristics are considered important. The cancer that develops in the animal model should be confined to the large intestine so that the development of the disease could be studied without confounding effects in other tissues. The histologic and molecular features should be similarly found in human colorectal cancer tissues. Additionally, the animal models should reflect the multifaceted intracellular pathways that are relevant to human colon cancer [3, 4].

Several colon cancer animal models have showed potentials for studying various initiating and environmental factors, specific dietary and genetic factors, as well as therapeutic options. These models can be categorized into induced and transgenic animal models, and each of these models differ in their relevance for studying various factors [3, 4]. Chemically or environmentally induced rodent model for colorectal cancer includes the administration of "Western diet", Heterocyclic amine, and alkylating agent such as N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methyl-N-nitrosourea (MNU) as well as 1,2-Dimethylhydrazine (DMH) or its metabolite Azoxymethane (AOM).

Each animal model offers its own advantages and disadvantages. The Western Diet model can be considered a good model for spontaneous colon cancer, but it requires a long period of induction and there is a lack of report on carcinogenesis steps in molecular level [5-6]. PhIP (2-amino-1-methyl-6-phenylimidozo [4,5-b] pyridine) is a heterocyclic amine byproduct from meat and fish cooking. In mice, PhIP induces formation of colonic aberrant crypt foci but not colon tumors. This model requires long period of induction with low tumor incidence rate [7-9]. MNNG and MNU are direct alkylating agents that induce colonic adenoma and carcinoma in a manner similar to histopathologic features of DMH induced model. Administration of both agents result in high incidence of colorectal cancer however these direct-acing carcinogens also induce neoplasia in organs other than colon, which turns into a confounding variable for this model [4].

DMH and its metabolite are most commonly used alkylating agents for sporadic CRC animal model. Repetitive treatment with this methylating agent was reported to produce colon tumors in rodents that exhibit many of the molecular and pathological features associated with the human sporadic CRC. In addition, this model offer advantages in term of cost, potency and convenience towards other chemical carcinogens [4, 10-14]. As a carcinogenic agent, DMH is becoming less readily available in Indonesia. This, in turn, requires that we search for comparable alternative. DMH metabolite, AOM, requires fewer metabolic activation step and is structurally closer to the ultimate carcinogen. Similar to DMH, AOM has been shown to promote histological and molecular changes that correspond with the orderly events' sequence that leads to the development of colorectal cancer. As the Wistar rats available at local vendors in Indonesia are not specific pathogen free animals, and with less defined genetic background, a pilot study is therefore necessary to investigate both AOM effect and appropriate dosage that will induce colon cancer in these animals.

Materials and Methods

Reagents

Azoxymethane, Hematoxylin, Eosin and Methylene blue were purchased from Sigma (St. Louis, MO, USA).

Animals

Forty-two adult male Wistar rats (100-120g) at six weeks of age were purchased from PT Indoanilab (Bogor, Indonesia). All rats were housed in conventional polypropylene plastic cages under controlled conditions (temperature of 25±2°C, humidity of 50±10%, and 12-hour light-dark cycles). The rats had free access to drinking water and were fed a basal diet containing 5% fat, 53% carbohydrate, and 25% protein (Laboratory Feed Industry, Bogor Agricultural University, Bogor, Indonesia). The rats were observed daily and their body weight was measured weekly.

CRC Induction

After three weeks acclimatization period (BW 120-150 g), the rats were randomly assigned into nine groups. Group 1, 4 and 7 served as negative control groups. Rats in these groups received saline treatment and were sacrificed at week 9, 17 and 25 respectively. Group 2, 5, and 8 were given two injections of AOM dissolved in normal saline, at 15 mg/kg BW subcutaneously within 1-week interval. Rats in these groups were sacrificed at week 9, 17 and 25 (8, 16 and 24 weeks after last AOM administration) respectively. Groups 3, 6 and 9 received two injections of AOM dissolved in normal saline, at 20 mg/kg BW subcutaneously; and were sacrificed at week 9, 17 and 25 (8, 16 and 24 weeks after last AOM administration). Euthanasia was performed by injection of Ketamine and Xylazine using three times the dose of Ketamine-Xylazine for anesthesia [15]. All studies were performed with the approval of the research ethics review committee of Mochtar Riady Institute for Nanotechnology (No. 001/MRIN-EX/ECL/I/2017).

Aberrant Crypt Foci (ACF) Identification

For ACF Analysis, we followed protocol as reported in Lu et al. [16]. Rats' colons were removed, rinsed in ice-cold 1X PBS pH 7.4, slit open longitudinally, and fixed flat between two filter papers which have been soaked in PBS. The colons were incubated in 10% neutral buffered formalin for 18 hours and subsequently stained with 0.2% methylene blue for 10 seconds. Methylene blue solution were prepared using the same formalin solution. Deeply stained crypts were inspected after staining, and the total number of ACF and the number of aberrant crypts (ACs) in each focus were counted under a light microscope at 100X magnification (Axioskop 40, Zeiss, Germany).

Histological Assay

All rats were examined grossly at necropsy. The intestine from the stomach to anus were removed and the large intestine were isolated.

The large intestine was slit open lengthwise, washed in saline and the mucosal surface was examined for gross pathology. Any lesions detected were measured, their location noted and the lesions were dissected. Normal appearing colons and a portion of the lesions were taken for histological examination, after fixation in 10% w/v formaldehyde overnight according to standard methods. After fixation, the lesions were embedded in paraffin, sectioned at 4 µm, stained with Hematoxylin and Eosin for evaluation under a light microscope at 10X and 40X Magnification (Axioskop 40, Zeiss, Germany).

Calculation of tumor incidence, number and volume

The tumor incidence, average tumor number and tumor size were calculated using formula as described by Jia et al. [17]. Tumor incidence calculated from the number of rats that developed tumors in the colon tissue after twice injection of AOM during the experiment.

Characterization and Calculation of dysplastic cells

Histopathological classification was based on criteria as described in [18]. In brief mild, moderate and severe dysplasia were categorized by the form of nuclei, cell polarity, structure of glandular epithelium, and the presence of goblet cells and immune cells in the mucosal layer. The presence of dysplastic cells was carefully noted in terms of classification, and number along 3 separate sections covering 10 areas of the colon tumor.

Statistical Analysis

For statistical analysis, values are expressed as means ± SD. All statistics were computed using SPSS21 (SPSS Software, SPSS Inc., Chicago, USA). Unpaired Student's t test and chi-square test were used to detect statistically significant differences between groups. A P-value of < 0.05 is considered significant.

Result

Physical parameters

The time course of the experiment and the animal treatment procedures are shown in Figure 1. Over the six-month period, all rats showed an increase in body weight. No significant difference in bodyweight gain was observed between the groups. Fig. 2 illustrates the body weight development during the entire duration of the experiment. Following AOM injections, the body weight of rats in both AOM groups was significantly lower compared to the control groups. At the end of 8 weeks, rats receiving AOM at 15 mg/kg BW and 20 mg/kg BW showed a decrease in body weight compared to rats in control groups (One-way ANOVA, *P* = 8E-3). At the end of 8 weeks after AOM injection, the mean body weight for control groups was 212.39±11.47 g, for AOM dose 15 mg/kg BW it was 184.08±10.61 g (post-hoc analysis, *P* = 0.077), and for rats receiving AOM 20 mg/kg BW the mean body weight was 155.61±8.01 g (post-hoc analysis, *P*=0.002). Similarly, at the end of 24 weeks, rats in AOM groups showed a lower bodyweight than control rats (One-way ANOVA, *P* = 0.0013). The mean body weight at the end of 24 weeks for control groups was 260.66±4.09 g, it decreased to 236.59±4.34 g for AOM dose 15 mg/kg BW (post-hoc analysis, *P* = 0.0012), and it further decreased to 222.84±15.28 g for rats receiving AOM 20 mg/kg BW (post-hoc analysis, *P*=0.0139). The body weight of rats in groups receiving AOM on average was lower than rats in control groups at the end of 16 weeks, however it was not statistically significant.

Tumor Incidence

Tumors were visualized in general by gross examination in colon tissue at the end of the experiment. Multiple nodules were detected macroscopically in the colonic mucosa of rats induced with both AOM doses with diameter ranging from 3 mm to 1.5 cm. The number of nodules in colonic mucosa of rats in the AOM groups are listed in Table 1.
Tumor incidence is determined by calculating the percentage of tumor-bearing rats from total rats in the group. Tumor-bearing rats are the number of rats with tumors developed in the colonic mucosa with the repeated injection of AOM during the whole experiment process. In this Pilot Study, both AOM doses resulted in 100% tumor incidence, as all rats treated with AOM developed tumor in the colonic mucosa in the course of the experiment. There was a statistically significant increase of mean tumor volume with longer induction period at AOM 15 mg/ kg BW. The mean tumor volume at 8 weeks post-AOM injection was 18.4±4.70 which increased to 35.45±10.46 mm^3 at 16 weeks and to 83.3±10.98 mm^3 at 24 weeks after the second AOM injection. No significant difference in tumor number and tumor volume was observed with longer induction period at AOM 20 mg/ kg BW. Figure 3 shows nodules detected in the colonic tissues of rats in AOM groups. Few nodules in colonic mucosa were detected in some rats in control groups. However, upon further observation, the nodules were identified as infiltrating lymphocyte to mucosal layer, and no dysplastic cells were detected (data not shown).

Aberrant Crypt Foci (ACF)

ACF are considered as potential pre-neoplastic lesions of the colon in both humans and experimental animals. ACF were distinguished from surrounding normal crypts by increased size, thickened epithelial cell lining, and enlarged cryptal area relative to surrounding normal crypts. Figure 4 shows normal cell colon and colon containing foci with 2, 3 and more than 4 aberrant crypts in rats treated with AOM.

Table 2 illustrates total number of ACF and number of foci that was detected in rats from all groups. ACF is further categorized by the number of aberrant crypts (1, 2, 3 or more than 4) formed in the foci. There was a significant difference in the number of ACF between 8 weeks and 16 weeks or 24 weeks experiment time (Fig.5A and B). In rats receiving two injections of AOM at 15 mg/kg BW, mean number of ACF after 8 weeks was 9.40±3.40, the number increased significantly to 31.60±2.16 after 16 weeks (*P* = 5E-4) and to 33.40±4.65 foci 24 weeks after AOM induction (*P* = 0.0031). Similarly, in rats receiving two injections of AOM at 20 mg/kg BW, 8 weeks after induction the mean number of ACF was 11.00±2.89, this number increased significantly to 30.40±5.12(*P* = 0.0109) and to 36.28±4.42 (*P* = 0.0033) at 16 weeks and 24 weeks post-AOM induction, respectively. However, within the same induction time, there was no statistically significant difference in the mean ACF number between the two AOM doses (Fig.5A and B).

Histology Evaluation

For further confirmation of the presence of dysplastic cells, Hematoxylin and Eosin staining were performed. In control animals, colon crypts were detected with goblet cells and normal colonocytes (Fig 6 A and B). Normal histopathological structure of the mucosal layer with glandular structure, an underlying sub-mucosa and muscular layer were observed in the tissue section of the animals. In rats treated with AOM, proliferation of colonocytes and decrease of glandular architecture of the crypts were observed indicating the presence of dysplastic cells. Mild dysplasia was detected with proliferation of lymphoid and glandular structure in the mucosa layer (Fig. 6C and 6D). Additionally, moderate dysplasia was identified with lymphoid proliferation in mucosal layer and degeneration of the glandular lining epithelium with loss of nuclei (Fig. 6E and 6F). Tissue section with severe dysplasia showed abnormal hyperplastic glandular in mucosa layer, degeneration of epithelial with loss nuclei and proliferation lymphoid in mucosa layer were detected (Fig. 6G and 6H). Moreover, inflammatory cell infiltration in the lamina propria of the mucosal layer with intact mucosal epithelium was also detected (Fig. 7)

Fig. 8 illustrates the mean number of mild, moderate and severe dysplastic cells in both AOM doses at various induction time. There was no statistically significant difference between mean number of dysplastic cells in various induction time in animals receiving AOM 15 mg/kg BW. At AOM dose 15 mg/kg BW, mean number of mild, moderate and severe dysplastic cells did not differ significantly at 8 weeks, 16 weeks and 24 weeks after AOM induction (Fig. 7A, $P > 0.05$). At AOM dose 20 mg/ kg BW, mean number of mild dysplastic cells increased from 31±0.707 at 8 weeks to 33±0.316 at 16 weeks after AOM induction ($P = 0.0325$). Similarly, the longer the induction period, the greater was the number of cells which was categorized as severe dysplastic. At 8 weeks after AOM induction, the mean number of cells was 27±1.048; this number increased to 30.4±0.979 ($P = 0.045$) and to 32±2.049 ($P = 0.073$) at 16 weeks and 24 weeks respectively.

Discussion

In this Pilot Study, we use Azoxymethane (AOM), a DMH metabolite as an alternative agent to induce CRC in male Wistar rats. Similar to DMH, AOM has been shown to induce colon cancer in a manner comparable to the pathogenesis of human sporadic colon cancer. After administration, AOM is metabolised into Methylazoxymethanol (MAM) by CYP2E1, which generates DNA mutations. Mutations associated with colon carcinogenesis have been reported in DMH/ AOM cancer model, for instance mutations in K-ras and β-catenin. As a result of these mutations, associated PI3K/Akt and MAPK pathways are activated, and β-catenin degradation is prevented which leads to cell proliferation. In addition, Transforming Growth Factor Beta (TGFβ), a protein that is essential for apoptosis, has been shown to be inactivated in AOM cancer model [19]. Despite the safety aspects associated with the use of carcinogens, and the considerable time required for tumour development, DMH or AOM induced CRC animal model is considered useful for the study of the molecular biology, prevention and treatment of colon cancer [20].

In this study, 42 male Wistar rats nine weeks of age were distributed into control, and two AOM groups. Animals in AOM groups received two subcutaneous injections of AOM dissolved in saline at 15 mg/kg BW or 20 mg/ kg BW within one week period. Animals in control groups received the equivalent volume of saline with the same frequency as animals in AOM groups. In general, treatment with both AOM doses resulted in lower body weight compared to control in all time point. As no significant difference between the groups was observed in food intake, the low body weight in AOM group rats is mainly due to the increased tumour burden.

Tumor induction using AOM in rodents were genetically dependent. Compared to Fischer F-344 rats, Wistar rats showed greater percentage of colorectal tumors, and the distribution of tumors in colorectum resembled more the distribution found in human pathology in DMH model [23]. In our Pilot Study, both AOM doses resulted in 100% tumor incidence in Wistar rats at 8, 16 and 24 weeks after AOM administration, similar to reported in [24]. Aberrant Crypt Foci (ACF) were one of the first observed biomarkers for murine CRC model. ACF in humans have been characterized for altered enzymatic activity, crypt dynamics and

proliferation; and they were found to closely resemble aberrant crypts seen in rodents treated with carcinogens [21]. In our experiment, treatment with both AOM doses induced formation of ACF, and we observed a considerable increase of total ACF in rats 16 weeks post-AOM injection compared to 8 weeks. ACF with multiple crypts (≥4) were considered more prone to progress into cancer, and were suggested to be better predictors for tumor incidence [22]. ACF ≥4 crypts were detected in our study. There was a statistically significant difference in number of ACF ≥4 crypts at 16 weeks and 24 weeks in comparison to 8 weeks after AOM administration. However, similar ACF number and ACF types were detected in rats 16 weeks and 24 weeks post-AOM treatment between the two AOM doses, suggesting that the formation of ACF in our experiment was not in a dose-dependent manner.

Histopathologic classification was based on the following criteria. Mild dysplasia was characterized as having elongated, crowded and pseudo-stratified nuclei with preserved polarity and a normal or slightly reduced number of goblet cells. Moderate dysplasia was characterized as having hyperchromatic proprieties and deformity of the cell nuclei, increased number of mitoses, thickening of the glandular epithelium and an increased number of immune (defence) cells in the connective tissue. Severe dysplasia was characterized as having broad, round or ovoid nuclei with prominent nucleoli, and atypical mitotic figures. In severe dysplasia, the nuclear polarity was partially lost and the number of goblet cells was significantly reduced or completely disappeared [18]. In this Pilot Study, colon of rats in control group showed predominantly normal crypts in which goblet cells and normal glandular structure were present. AOM administration in both 15 mg/ kg BW and 20 mg/ kg BW doses resulted in the formation of mild, moderate and severe dysplastic cells starting from 8 weeks after last AOM injection. While number of severe dysplastic cells increased with longer period of induction at AOM 20 mg/ kg BW, there was no statistically significant difference between mean number of dysplastic cells in various induction time in animals receiving AOM 15 mg/kg BW.

In conclusion, administration of AOM as low as 15 mg/kg BW SC is able to induce colon carcinogenesis in male Wistar rats. Higher dose is not necessary since it does not result in higher tumor incidence. The development of colon carcinogenesis in the AOM rat model involves the formation of ACF lesion, mild, moderate and severe dysplastic cells which starts at 8 weeks and peaks at 16 weeks after AOM administration. Results of this pilot study can pave the way for further studies on the elucidation of Colorectal Cancer (CRC) molecular pathogenesis and the discovery of preventive and therapeutic agents for the disease.

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References

1. Kwong LN, Dove WF. APC and its modifiers in colon cancer. Adv Exp Med Biol.(2009) 656, 85-106

2. Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, Bray F (2018). Global Cancer Observatory: Cancer Today. Lyon, France: International Agency for Research on Cancer. Available from: https://gco.iarc.fr/today, accessed [5 January 2020].

3. Johnson RL and Fleet JC. Animal models of colorectal cancer. Cancer Metastasis Rev. (2013)32(0): 39–61.

4. Perše M, Cerar A. Morphological and molecular alterations in 1,2 dimethylhydrazine and azoxymethane induced colon carcinogenesis in rats. J Biomed Biotechnol. 2011; 2011:473964.

5. Newmark HL, Yang K, Lipkin M, Kopelovich L, Liu Y, Fan K, Shinozaki H. A Western-style diet induces benign and malignant neoplasms in the colon of normal C57Bl/6 mice. Carcinogenesis. 2001 Nov;22(11):1871-1875.

6. Newmark HL, Yang K, Kurihara N, Fan K, Augenlicht LH, Lipkin M. Western-style diet-induced colonic tumors and their modulation by calcium and vitamin D in C57Bl/6 mice: a preclinical model for human sporadic colon cancer. Carcinogenesis. 2009 Jan;30(1):88-92.

7. Tanaka T, Suzuki R, Kohno H, Sugie S, Takahashi M, et al. Colonic adenocarcinomas rapidly induced by the combined treatment with 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and dextran sodium sulfate in male ICR mice possess beta-catenin gene mutations and increases immunoreactivity for beta-catenin, cyclooxygenase-2 and inducible nitric oxide synthase. Carcinogenesis. (2005) 26:229–238.

8. Nakagama H, Nakanishi M, Ochiai M. Modeling human colon cancer in rodents using a food-borne carcinogen, PhIP. Cancer Sci. (2005) 96:627–636.

9. Andreassen A, Vikse R, Mikalsen A, Adamovic T, Steffensen IL, Hjertholm H, Levan G, Alexander J. 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induces genetic changes in murine intestinal tumours and cells with ApcMin mutation. Mutat Res. (2006) 604 (1-2):60–70.

10. Jucá MJ, Bandeira BC, Carvalho DS, and Leal AT. Comparative study of 1,2-dimethylhydrazine and azoxymethane on the induction of colorectal cancer in rats. Journal of Coloproctology (Rio de Janeiro), (2014). 34(3), 167-173.

11. Christudoss P, Selvakumar R, Pulimood AB, Fleming JJ, Mathew G. Zinc and zinc related enzymes in precancerous and cancerous tissue in the colon of dimethyl hydrazine treated rats. Asian Pac J Cancer Prev. (2012) 13(2):487-492.

12. Kim JM, Park E. Coenzyme Q10 attenuated DMH-induced precancerous lesions in SD rats. J Nutr Sci Vitaminol (Tokyo). 2010;56(2):139-144.

13. Aroch I, Kraus S, Naumov I, Ron E, Shapira S, et al Chemopreventive effects of Coltect, a novel dietary supplement, alone and in combination with 5-aminosalicylic acid in 1,2-dimethylhydrazine-induced colon cancer in rats. Therap Adv Gastroenterol. 2010 Sep;3(5):281-289.

14. DE-Souza ASC, Costa-Casagrande TA. Animal Models for Colorectal Cancer. Arq Bras Cir Dig. 2018; 31(2): e1369. doi: 10.1590/0102-672020180001e1369.

15. American Veterinary Medical Association. American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition. Version 2013.0.1. ISBN 978-1-882691-21-0.

16. Lu, Q., Jiang, B., Lin, C. et al. Dark Aberrant Crypt Foci with activated Wnt pathway are related to tumorigenesis in the colon of AOM-treated rat. J Exp Clin Cancer Res (2008) 27, 26.

17. Jia Y, Xu G, Zhou W, Wang Z, Meng L, et al. (2014) Diabetes Promotes DMH-Induced Colorectal Cancer by Increasing the Activity of Glycolytic Enzymes in Rats. PLoS ONE 9(10): e110455.

18. Paulsen JE, Løberg EM, Olstørn HB, Knutsen H, Steffensen IL, Alexander J. Flat dysplastic aberrant crypt foci are related to tumorigenesis in the colon of azoxymethane-treated rat. Cancer Res. 2005 Jan 1;65(1):121-9.

19. Chen J and Huang XF. The signal pathways in azoxymethane-induced colon cancer and preventive implications. Cancer Biology & Therapy (2009), 8:14, 1313-1317,

20. Workman P, Aboagye EO, Balkwill F, Balmain A, Bruder G, et al. Guidelines for the welfare and use of animals in cancer research. British Journal of Cancer (2010) 102, 1555 – 1577

21. Cheng L and Lai MD. Aberrant crypt foci as microscopic precursors of colorectal cancer. World J Gastroenterol. 2003 Dec 15; 9(12): 2642–2649

22. Zhao Y, Hasjim J, Li L, Jane JL, Hendrich S, Birt DF. Inhibition of azoxymethane-induced preneoplastic lesions in the rat colon by a cooked stearic acid complexed high-amylose cornstarch. J Agric Food Chem. 2011 Sep 14;59(17):9700-8

23. Martina Perše and Anton Cerar. The dimethylhydrazine induced colorectal tumours in rat - experimental colorectal carcinogenesis. Radiol Oncol 2005; 39(1): 61-70.

24. Željka Večerić and Anton Cerar. Comparison of Wistar vs. Fischer rat in the incidence of 1,2-dimethylhydrazine induced intestinal tumors. Radiol Oncol 2004; 38(3): 227-34.

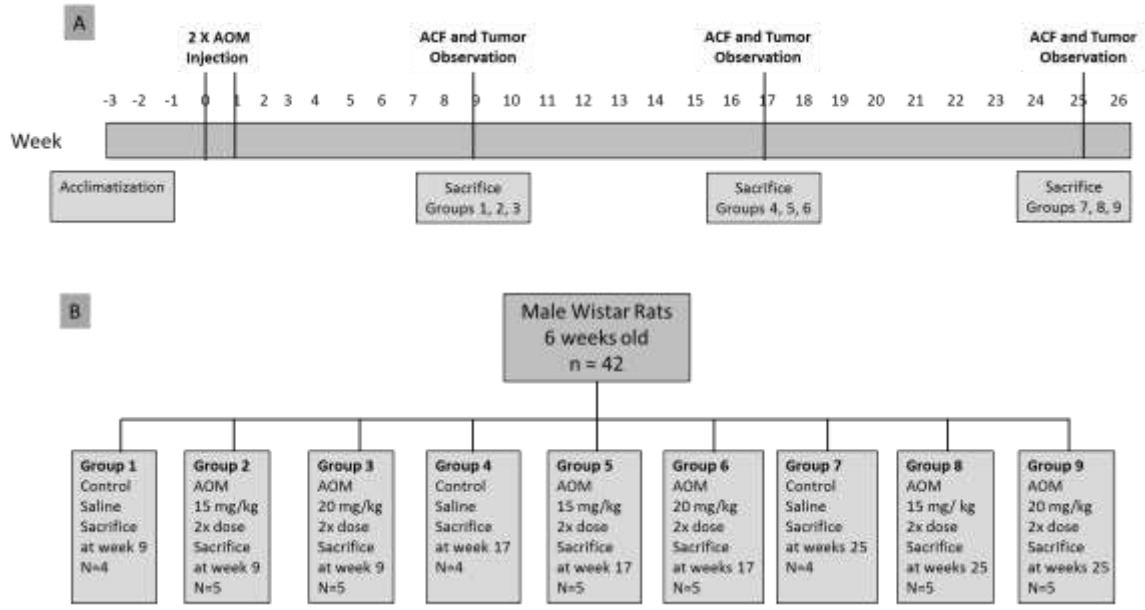

Fig.1. **Experimental Outline.** A. Timeline for AOM administration and sacrifice. B. Experimental group distribution. AOM was administered subcutaneously. AOM: Azoxymethane; ACF: Aberrant Crypt Foci

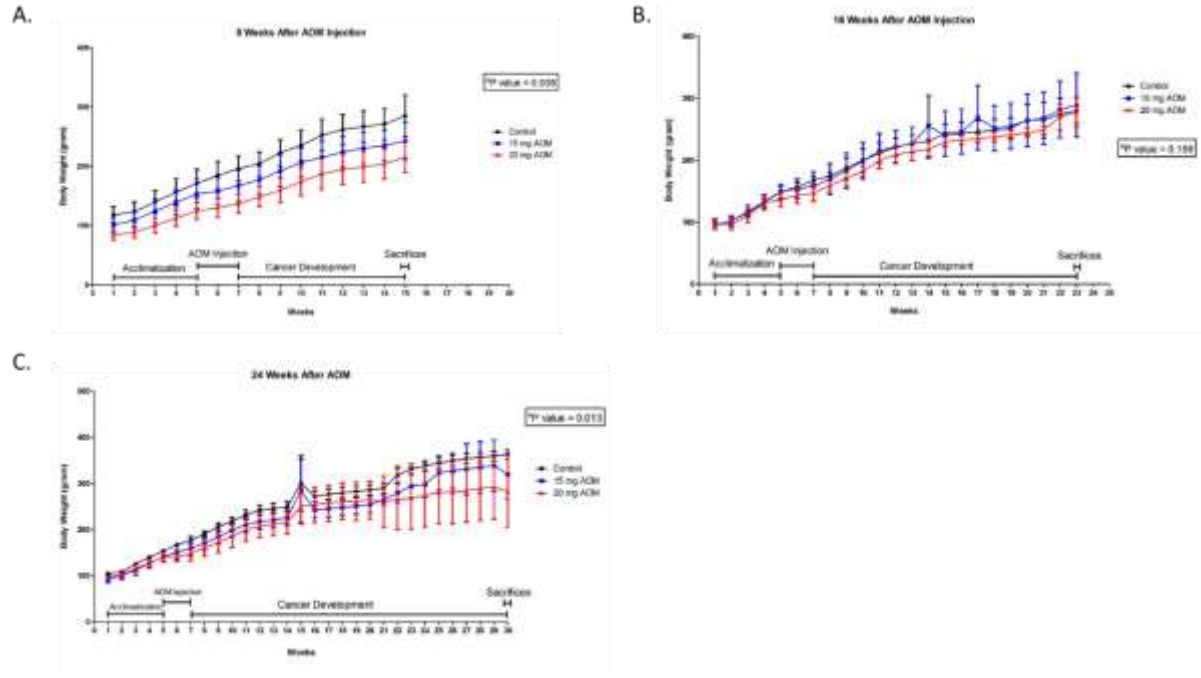

Fig.2. **Body weight development of rats in control and AOM treated groups.** A. 8 weeks- B. 16 weeks- C. 24 weeks- post AOM injection. Statistical Analysis using One-way ANOVA, $P < 0.05$ is considered significant.

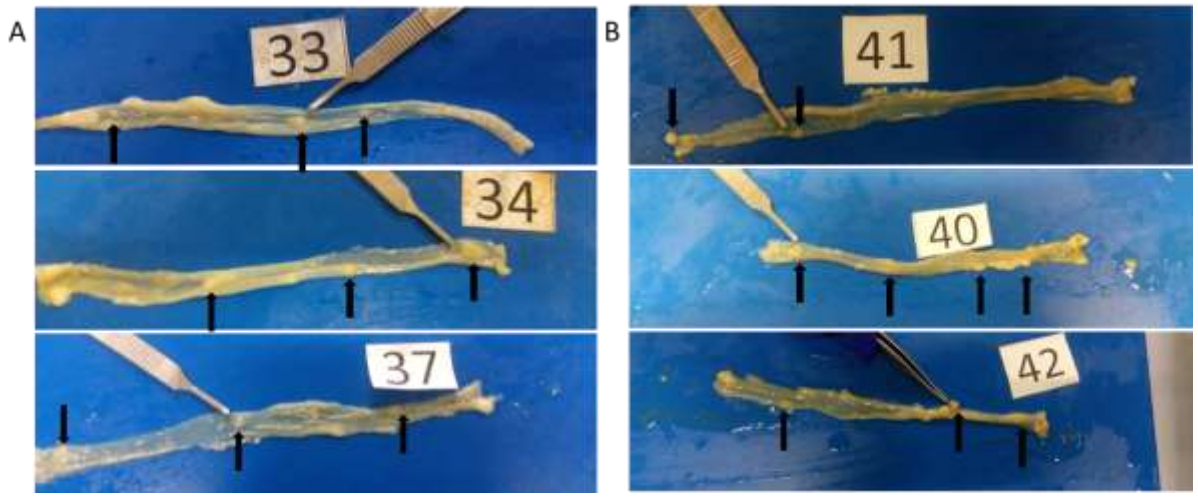

Fig.3. Nodules in colon of rats in AOM groups. Arrows indicate the presence of mucosal nodule A. 24 weeks after 2x injection of AOM 15 mg/kg BW B.24 weeks after 2X injection of AOM 20 mg/kg BW

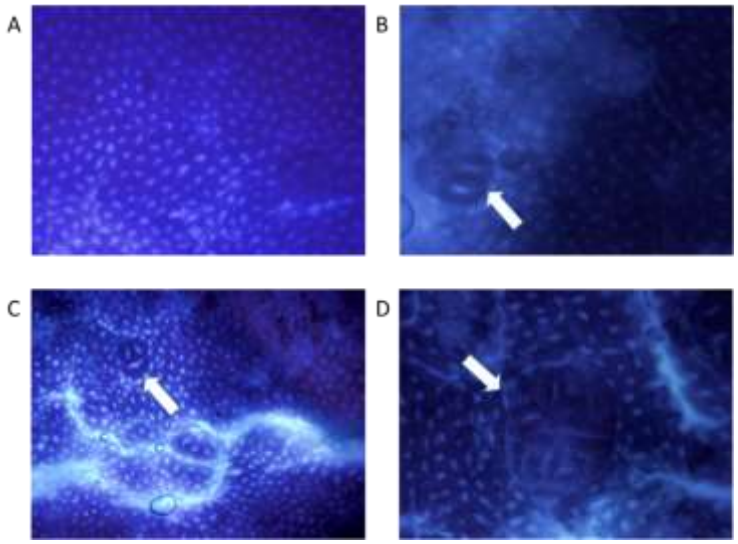

Fig.4. **ACF formation in control and AOM-induced animals.** A. Control group; Foci containing B. 2 aberrant crypts: C. 3 aberrant crypts: D. more than 4 aberrant crypts in rat treated with AOM 15 mg/ kg BW 16 weeks after AOM induction

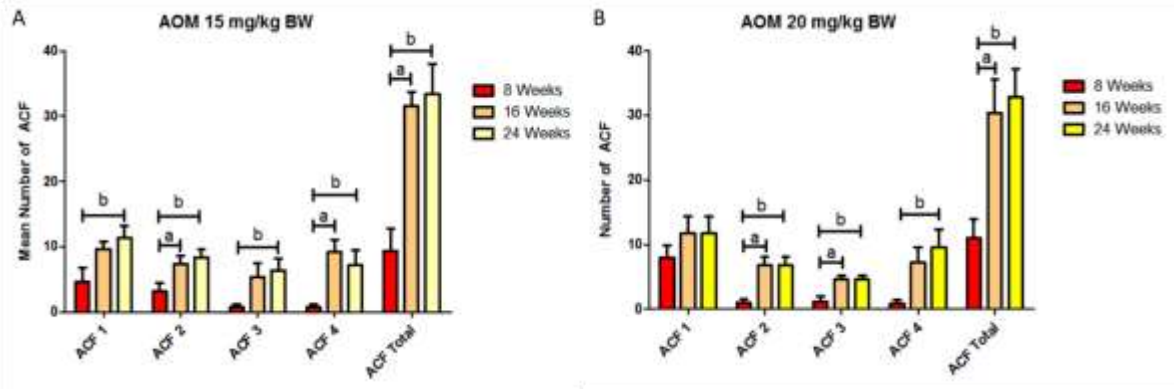

Fig.5. Total and average **number and type of ACF in AOM-induced animals.** A. 15 mg/kg BW. B. 20 mg/kg BW. Statistical analysis: a. t-test, $P < 0.05$, 8 weeks vs 16 weeks, b. t-test, $P < 0.05$, 8 weeks vs 24 weeks

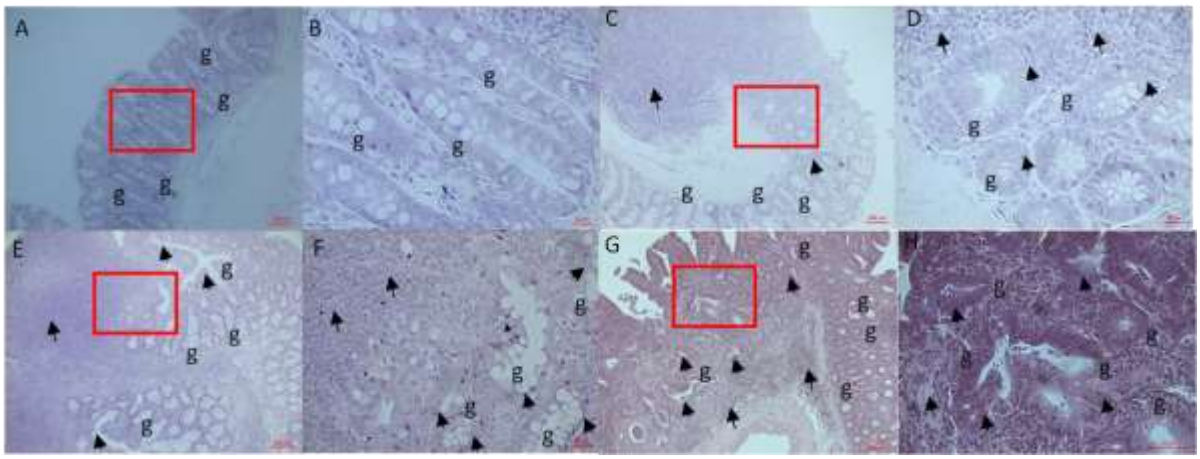

Fig. 6. Representative section of colon architecture histology under the light microscope after haematoxylin and eosin staining. A, B: Normal crypt of rat colon observed in control group. g = glandular structure. C, D: Mild dysplastic cells observed in rat's colon after induction with AOM 20 mg/kg BW. Arrows indicate lymphoid proliferation in mucosal layer. Arrowheads show proliferation of glandular structure in the mucosa layer. E, F: Moderate dysplasia. Arrows show lymphoid proliferation in mucosal layer. Arrowheads indicate degeneration of the glandular lining epithelium with loss of nuclei. G, H: Severe dysplasia observed in rat's colonic mucosa after treatment with AOM 15mg/kg BW; Arrows indicate lymphoid proliferation in mucosa layer; Arrowheads show proliferation glandular structure with loss of nuclei. (A, C, E, and G: 10X Magnification; B, D, F, and H: 40X Magnification)

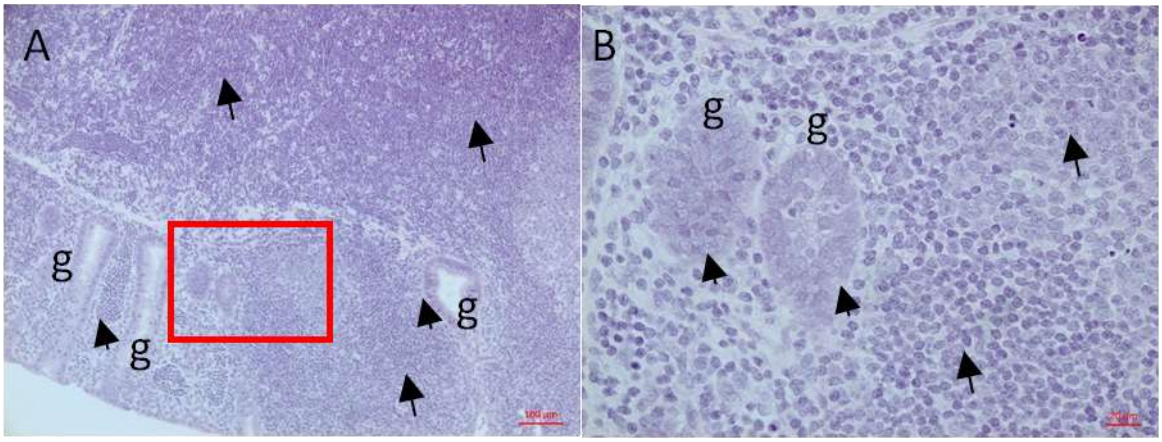

Fig. 7. Moderate dysplasia with inflammatory cell infiltration in the the mucosal layer. Arrow: lymphocyte cells. Arrowheads: glandular proliferation. A: 10X, B: 40X Magnification

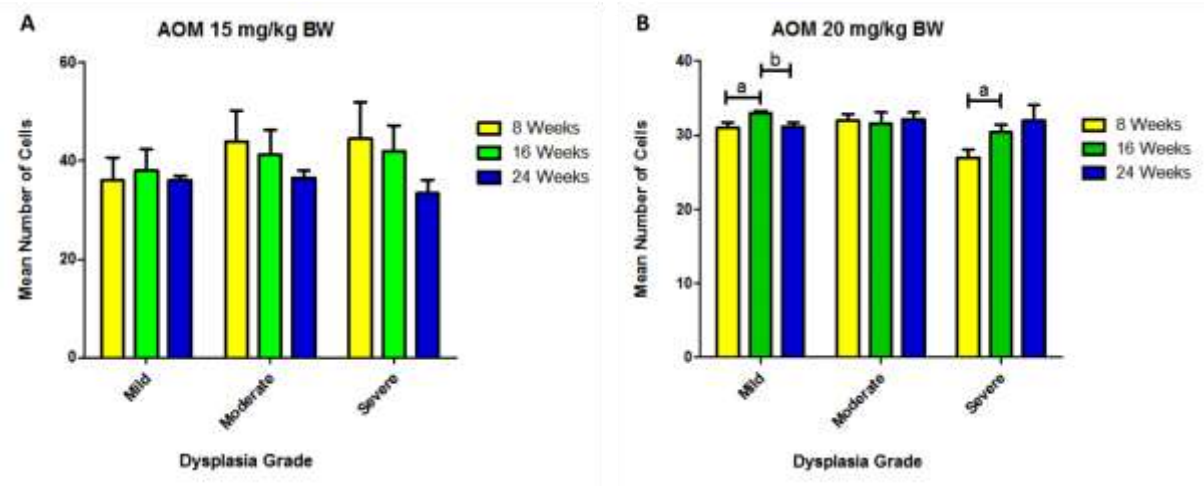

Fig. 8. Mean number of cells with different Dysplasia grade in rats induced with (A) AOM 15 mg/kg BW and (B) 20 mg/kg BW. Statistical analysis: a. t-test, $P < 0.05$, 8 weeks vs 16 weeks, b. t-test, $P < 0.05$, 16 weeks vs 24 weeks. There was no statistically significant difference between mean number of dysplastic cells in various induction time in animals receiving AOM 15 mg/kg BW.

Table 1. Incidence, number and volume of tumors

	Group					
AOM dose	15 mg/ kg BW			20 mg/ kg BW		
	8 wks	16 wks	24 wks	8 wks	16 wks	24 wks
n	5	5	5	5	5	5
Tumor-bearing rats	5	5	5	5	5	5
Tumor incidence	100%	100%	100%	100%	100%	100%
Number of tumors	18	12	21	18	19	16
Average tumor number	3.6 ± 0.75	2.4 ± 0.75	4.2 ± 0.66	3.6 ± 0.75	3.8 ± 0.66	3.2 ± 0.49
Tumor Volume (mm^3)	18.4±4.70	35.45±10.46	83.3±10.98[a,b]	64.3±14.00	72.4±13.89	45.3±6.89

Values are expressed as mean ±SEM. Average tumor number and tumor volume were analyzed using unpaired student's T-test. [a] *P* = 6E-4 (8wks vs 24 wks); [b]1*P* = 0.013 (16 wks vs 24 wks)

Table 2. AOM-induced ACF in rats' colon

Group	**n**	**total number of ACF/ rat**	**Number of foci containing**			
			1 crypt	2 crypts	3 crypt	≥4 crypts
Control 8 wks	4	0	0	0	0	0
Control 16 wks	4	0	0	0	0	0
Control 24 wks	4	0	0	0	0	0
AOM 15 mg/kg BW 8 wks	5	9.40±3.40	4.60±2.18	3.20±1.24	0.80±0.37	0.80±0.37
AOM 15 mg/kg BW 16 wks	5	31.60±2.16	9.60±1.20	7.40±1.24	5.40±2.11	9.20±1.93
AOM 15 mg/kg BW 24 wks	5	33.40±4.65	11.40±1.80	8.40±1.60	6.40±1.77	7.20±2.22
AOM 20 mg/kg BW 8 wks	5	11.00±2.89	8.00±1.92	1.00±0.54	1.20±0.80	0.80±0.58
AOM 20 mg/kg BW 16 wks	5	30.40±5.12	9.60±1.02	6.20±1.01	4.40±0.40	7.50±3.12
AOM 20 mg/kg BW 24 wks	5	36.28±4.42	11.80±2.51	6.80±1.28	4.60±0.60	9.60±2.80

Values are expressed as mean±SEM. Average ACF and aberrant crypts number were analyzed using unpaired student's T-test