RESEARCH ARTICLE

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Morphological Changes and Inflammation Preceded the Pathogenesis of 1,2-Dimethylhydrazine-Induced Colorectal Cancer

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Abstract

Objective: This study examined the morphological changes in the colonic mucosa and the presence of inflammation in rats induced with 1,2-dimethylhydrazine (DMH) 30 mg/kg BW over 9, 11, and 13 weeks without a latency period. **Methods:** Hematoxylin and eosin staining was performed to assess the morphology and characteristic alteration of the epitheliocytes in the colon. Immunohistochemistry was employed to assess the expression of tumor necrosis factor (TNF)-α and cyclooxygenase-2 (COX-2). The difference in the severity of inflammation and COX-2 expression was examined using one-way analysis of variance. The correlation of COX-2 expression with the severity of inflammation was analyzed using Spearman's rank correlation test. **Result:** Until week 13, chronic inflammation and non-hyperplastic and hyperplastic aberrant crypt foci occurred. The severity of inflammation gradually shifted from high moderate to low moderate. TNF-α expression was high in all groups; however, COX-2 expression was gradually lower with longer duration of induction, which corresponded with the severity of inflammation. **Conclusion:** DMH induction until week 13 without a latency period caused chronic inflammation without the formation of adenoma or adenocarcinoma. A very strong correlation was established between COX-2 expression and inflammation.

Keywords: CRC (colorectal cancer)- dimethylhydrazine- aberrant crypt foci- cyclooxygenase-2- tumor necrosis factor-α

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Introduction

Colorectal cancer (CRC) is the third common cancer worldwide, mainly in industrialized countries, and is considered the second common cause of cancer-related death, which accounted for approximately 0.9 million deaths in 2020 [1]. Recently, the incidence of CRC has drastically increased in Asia. Approximately 70% of CRC cases were sporadic, which were affected by environmental factors such as red meat, high-fat diet, low-fiber diet, smoking, alcohol consumption, and sedentary lifestyle. Only 5%–10% of CRC cases are caused by inherited mutations in cancer-related genes, and 25% of patients have a family history [2].

Rodents can be good learning models of colorectal malignancies because of their high similarity to humans in many aspects. [3]. To generate a pathological feature of colorectal tumor similar to the sporadic type of CRC in humans, rats were chemically induced with 1.2 dimethylhydrazine (DMH) [4–8]; therefore, it is a reliable and reproducible experimental model for studying sporadic (nonfamilial) CRC [4]. The duration of CRC induction using DMH varied from 8 h to 78 weeks, and cancer-associated changes can be seen from week 5 after DMH induction [7]. A study showed that aberrant crypt foci (ACF) formed after 4 weeks of DMH induction, whereas ACF further transformed into dysplasia after 30 weeks of induction [9]. Intraperitoneally, DMH generated preneoplastic lesion with long-term induction, such as 17 weeks [8]. Subcutaneous induction for 12 weeks triggered tumor formation when combined with 24 weeks of a latency period [10]. The histological section of the

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colon of rats subcutaneously induced for 5 weeks and then euthanized at week 14 showed massive infiltrative inflammation [5]. Subcutaneous injection of DMH caused 100% epithelial dysplasia and precancerous lesions after 12 weeks. Exposure to DMH for several weeks caused histopathologic and metabolic changes in the colonic mucosa [7].

DMH induction can increase the severity of dysplasia from mild, moderate, severe, and carcinoma in situ. DMH is an effective and efficient agent because it can trigger the development of preneoplastic and inflammatory lesions in the developmental sequence from adenoma until more advanced stages [5–8].

Inflammation is one of the preneoplastic cancer lesions in the pathogenesis of CRC. Multiple inflammatory factors such as cytokines, immune cells, and other immune mediators have been discovered to play crucial roles in the initiation, growth, and metastasis of CRC. Inflammation has been associated with several CRC risk factors, i.e., obesity, smoking, diet, diabetes, microbiome [3]. Inflammatory cells present in the tissue produces reactive oxygen species (ROS) and nitrogen species, which increased the expression of several genes related to carcinogenesis, such as p53, DNA MMR protein, and DNA base excision repair protein, and transcription factor NF-κB or signaling protein cyclooxygenase-2 *(COX-2)* [11].

COX is a catalytic enzyme that converts arachidonate to prostaglandin E2, a regulatory compound in cell proliferation, angiogenesis, and inflammation. These process are pivotal in tumorigenesis [12, 13]. COX-2 is produced under inflammatory conditions and responded by proinflammatory cytokines such as interleukin-1α/β (IL-1α/β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). A hypoxic environment will stimulate factor 1 alpha and the expression of COX-*2* in CRC [14, 15]. COX-2 was also linked to epithelial premalignant and malignant lesions in different segments of the gastrointestinal tract [12].

TNF-α is a key contributor in the inflammation in CRC and mediates cell signaling processes required in the immune response and carcinogenesis through its interaction with TNFR1 and/or TNFR2. This interaction induces NF-κB to trigger signal transduction. NF-κB is a transcription factor with multiple roles in various biological activities, such as inflammation, cell survival, proliferation, angiogenesis, tumor promotion, and metastasis. NF-κB links inflammatory signaling and cancer and is involved in nearly every stage of cancer development, including invasion and metastasis [16].

This preliminary study evaluated changes in the mucosal colon structure of Sprague–Dawley rats after the administration of DMH at 30 mg/kg BW for 9, 11, and 13 weeks and examined whether the induction can trigger the development of adenoma or adenocarcinoma. In addition to the mucosal colon structure, the morphology and characteristics of epithelial cells of the colon were also examined. The presence of inflammatory cells in the mucosal and submucosal layer was examined to determine the degree of inflammation. The expression levels of TNF-α and COX-2 were also examined, and the correlation of those parameters with the severity of inflammation was analyzed.

Materials and Methods

Animals, DMH induction, and organ colon collection

Thirteen Sprague–Dawley rats, weighing 170–220 g, were used in this study. Twelve rats were divided into three groups of 4 (week 9, week 11, and week 13 groups), and one control rat was untreated. The rats were quarantined and acclimatized for 7 days before the induction. During the study, the rats were kept in animal rooms with a temperature of 23 ± 2 °C, relative humidity of 75% ± 15%, and light–dark cycle of 12:12 h. The rats were housed in groups of four animals per cage (size, 428 × 267 × 210 mm). The cages were placed in a quiet room with minimized noise. The rats were fed ad libitum with AD II and reverse-osmosis water. After 7 days of acclimatization, all groups were injected with DMH (QR4PB-PG, TCI®, Tokyo Chemical Industry CO.LTD, Tokyo, Japan) at 30 mg/kg BW subcutaneously once a week at weeks 9, 11, and 13. Before administration, DMH was diluted in a saline solution containing 0.9% of NaCl (Otsuka®, Indonesia).

The laboratory animal care protocol in this study followed animal ethics guidelines. This study was approved by the Animal Ethics Committee of the Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia, with Ethical Committee Approval no. "No.93/EC/H/FK-UNDIP/IX/2020."

After the induction period (weeks 9, 11, and 13), the rats were sacrificed using the combination of ketamine (100 mg/kg BW) and xylazine (10 mg/kg BW) injected intramuscularly as an anesthesia and were then decapitated. The rats were laid down in the supine position with straight feet. The ventral body until the inguinal region was disinfected using 70% alcohol. Incision was made in the midline from the xiphoid process until the pecten ossis pubis. This incision exposed the entire intra-abdominal cavity. Colon organs were cut transversely 0.5 cm from the upper border of the rectum as long as 0.5 cm. The organ was washed with phosphate-buffered saline and directly placed in 10% buffer formalin. After 24 h, the fixed colon tissues were processed into formalin-fixed paraffin-embedded (FFPE) tissue according to the standard protocol. FFPE tissue blocks were sliced at a thickness of 4 μm for hematoxylin and eosin (H&E) staining and immunohistochemistry.

H&E staining for morphology and inflammation examination

H&E staining was performed according to standard protocol [17]. The morphology and characteristic alteration of the epithelial cells in the colon were described, including the mucosal damage and presence of ACF. Epithelial cell alteration was observed in five fields of view. Inflammatory cells were observed in the colonic mucosa, the amount and distribution of inflammatory infiltrates were determined by eyeballing observation in four rats, and the severity of inflammation was scored using the modification of the method described by Erben et al. [18], as shown in Table 1 [18]. A light microscope

(OLYMPUS model CX21FS5) was used to observe the staining result, and images were captured using Optilab Upgrade Advance and Optilab Viewer 2.2 software.

Immunohistochemistry staining

Sliced tissue sections were deparaffinized and rehydrated. The expression levels of COX-2 and TNF-α were examined using antibody anti-COX-2 (PA5-17614, Invitrogen) and antibody anti-TNF-α (ab6671, Abcam), respectively. Detection was performed according to the protocol from the Mouse and Rabbit Specific HRP/DAB IHC Detection Kit Micro-polymer (ab236466, Abcam). The immune-positive result for TNF-α was examined by visual observation of the whole area; when the immunopositive cell were >10%, the result was positive [19]. Immunopositive cells (>10% positive cells) were further scored based on the staining intensity: 1 = weak, 2 = moderate, and 3 = strong. The immunopositive result for COX-2 was examined using the immunoreactive score (IRS). The percentage of positive cells was scored 0–4 and staining intensity was scored 0–3. The final score was obtained by multiplying the score of positive cells and the staining intensity score (Table 2) [20]. A light microscope (OLYMPUS model CX21FS5) was used for observation, and images were captured using Optilab Upgrade Advance and Optilab Viewer 2.2 software.

Statistical analysis

The difference in inflammation severity and COX-2 expression was examined using the one-way analysis of variance, and $p < 0.05$ was considered statistically significant. The correlation of COX-2 expression with the severity of inflammation was analyzed using Spearman's rank correlation test. The R value was used to evaluate the level of correlation between those parameters.

Results

Morphology Alteration and Inflammation of the Colonic Mucosa

Figure 1 represents H&E-stained sections of the colon of week 9, 11, and 13 DMH-induced rats. The colon of the untreated rats had intact mucosa (Figure 1A). In general, most of the colon of all DMH-induced rats showed normal mucosa with good differentiation. The boundary of each layer in the colonic wall was clear. However, inflammatory infiltration can be observed in different levels among the groups, and we did not distinguish the type of the inflammatory cells. Severe inflammation was observed in the colon of week 9 DMH-induced rats, with an average score of 2.25 (moderate–strong), followed by the colon of week 11 DMH-induced rats with a score of 2 (moderate), and the mildest inflammation was observed in the colon of week 9 DMH-induced rats with a score of 1.5 (mild–moderate). At different time points of induction, the severity of inflammation significantly varied from moderate–strong, moderate, to mild–moderate at weeks 9, 11, and 13, respectively ($p = 0.023$) (Table 3 and Figure 2). Severe inflammation was also indicated by the abundance of lymphoid nodules in the mucosal layer in one of the rats (Figure 1D). In all colonic walls of the treated rats,

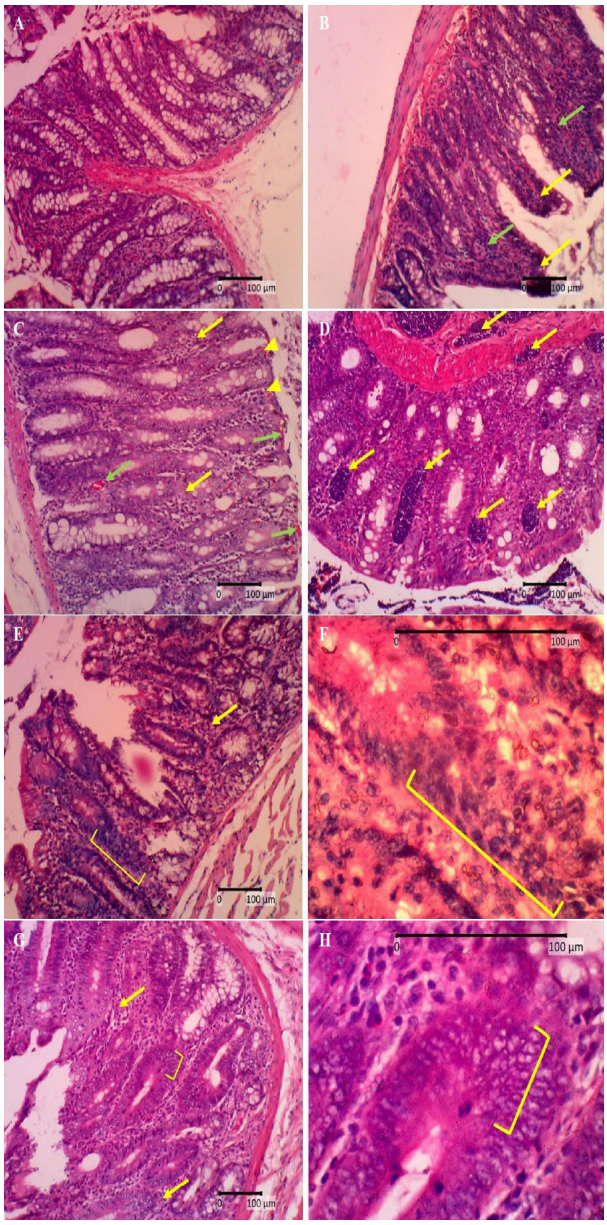

Figure 1. Morphology of the Colon Mucosa of DMH-induced Sprague–Dawley Rats (hematoxylin and eosin staining). (A–E, G) 100x magnification. (F & H) 400x magnification. (A) Normal control, colon of the uninduced rat. (B) Colon of a week 9 DMH-induced rat, showing inflammatory cells in between the epithelial cells (yellow arrow) and lamina propria (green arrow). (C) Colon of a week 9 DMH-induced rat, showing inflammatory cells in between the epithelial cells (yellow arrowhead) and the lamina propria (yellow arrow) and hemorrhage in the mucosal surface (epithelium and lamina propria; green arrow). (D) Heavy inflammation in the mucosal and submucosal layer with numerous lymphatic nodules (yellow arrow) in week 9 DMH-induced rats. (E) Colon of a week 11 DMH-induced rat, showing inflammatory cells in the lamina propria (yellow arrow) and aberrant crypt foci (ACF, yellow line). (F) 400x magnification of the ACF in the colon of a week 11 DMH-induced rat (yellow line). (G) Colon of a week 13 DMH-induced rat, showing inflammatory cells in the lamina propria (yellow arrow) and epithelial crypt hyperplasia (marked with yellow line). (H) 400x magnification of the epithelial crypt hyperplasia in the colon of a week 13 DMH-induced rat (yellow line). Scale bar: 100 µm.

Table 1. Modification of the Inflammatory Cell Infiltrate Scoring Method Described by Erben et al. (2014)

Description	Semiquantitative Score	Score number
10-25% scattered infiltrate cells and only found in one layer (epithelium or lamina propria or muscularis mucosae or submucosa)	Mild	1
26-50% infiltrate cells and found in one or two layers	Moderate	2
>51% dense infiltrate cells and found in more than 2 layers	Marked or Strong	3

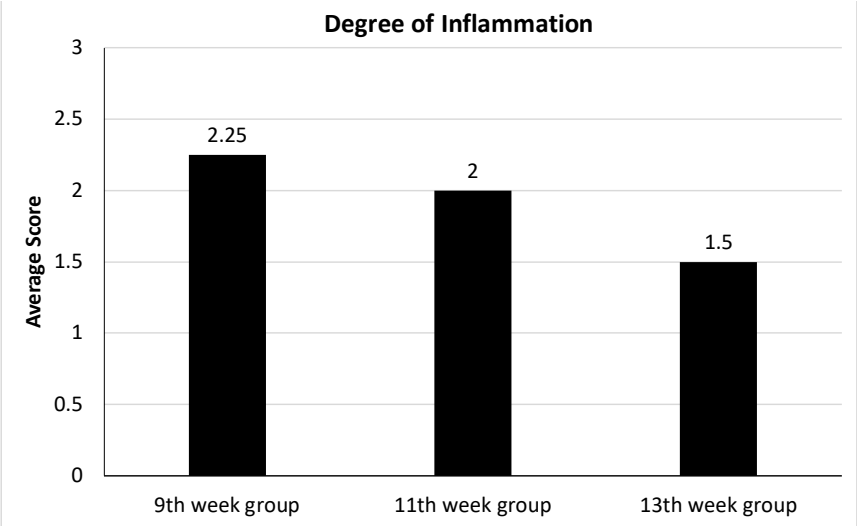

Figure 2. Degree of Inflammation in the Colon of DMH-Induced Rats. The level of inflammation in the colon of the rats in 9th week group was moderate-strong with score 2.25, 11th week group was moderate with score 2, and 13th week group was mild-moderate with score 1.5. The severity of inflammation was significantly different in all groups (p=0.023).

Table 2. Measurement Method of the Immunoreactivity Score (IRS) Described by Fedchenko and Reifenrath (2014) [20]

A (% of positive cells)	B (intensity of staining)	Final IRS score (multiplication of A and B)
0 = no positive cells	0 = no color reaction	0-1 = negative
1 = <10% of positive cells	1 = mild reaction	2-3 = mild
2 = 10-50% of positive cells	2 = moderate reaction	4-8 = moderate
3 = 51-80% of positive cells	3 = intense reaction	9-12 = strongly positive
4 = >80% of positive cells	Final IRS score (A x B): 0-12	

inflammatory infiltration can be found in between the epithelial cells and the lamina propria (Figure 1B–1D).

Changes in the mucosa cells were observed in the treated rats. ACF can be found in the colon of week 11 and 13 DMH-induced rats (Figure 1E and 1F) and colon of a week 13 DMH-induced rat; other than the ACF, crypt epithelial cell hyperplasia was also be observed. The crypt thickening was caused by the increasing number of epithelial cells in the crypt, which are seen in multiple layers (Figure 1G and 1H). The longer the induction, the milder the inflammation, and the higher the risk of the epithelial cells of the colon to hyperplasia.

TNF-α and COX-2 expression and the correlation with the severity of inflammation

Figure 3 represents the expression of TNF-α in the cytoplasm of cells in the rat colon. In general, the expressions were demonstrated as diffused staining with equally strong intensity in all colonic walls of the DMH-induced rats.

Figure 4 presents the expression of COX-2 on the rat colon. COX-2 staining was observed in the cell cytoplasm.

Table 3. Severity of Inflammation

Sample	Average score	Degree of Inflammation	p value
9th week group	2:25	moderate - strong	
11th week group	2	moderate	0.023
13th week group	1:05	mild - moderate	

*One-way ANOVA, p < 0.05 was considered statistically significant.

Table 4. IRS Score of COX-2 Expression

Sample	Average of IRS Score	Note	P value
9th week group	9	Strong	
11th week group	5	Moderate	<0.0044
13th week group	4	Moderate	

*One-way ANOVA, p < 0.05 was considered statistically significant

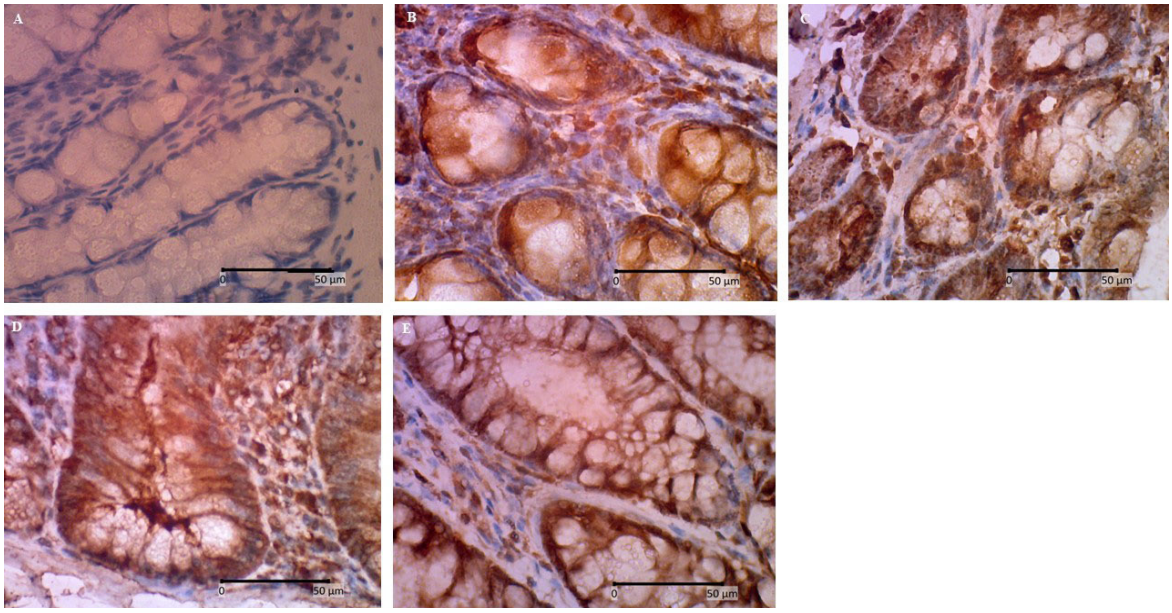

Figure 3. Expression of Tumor Necrosis Factor (TNF)-α in the Rat Colon Mucosa. (A) Negative control staining was performed in the colon section of the untreated rats without antibody anti-TNF-α. The negative control showed none of the immunopositive cells. (B) Positive control staining was performed in the colon section of the untreated rats with antibody anti-TNF-α. Immunopositive cells showed cytoplasmic brown staining. (C) Week 19 DMH-induced rat colon. (D) Week 11 DMH- induced rat colon. (E) Week 13 DMH-induced rat colon. The distributions of TNF-α staining in (C), (D), and (E) were diffused with high staining intensity (scored 3). All figures were captured at 400x magnification. Scale bar: 50 µm.

Table 5. Correlation between COX-2 Expression and Degree of Inflammation

Parameter	Degree of inflammation	
	R	p value
COX-2 expression	0.456	0.146

*Strength of correlation coefficient value: 0.00–0.25 (very weak); 0.26–0.50 (moderate); 0.51–0.75 (strong); and 0.75–1,00 (very strong).

Immunopositive-staining cells were observed in >80% of the colon from all groups, with varying intensities among the groups from mild to intense reactions. Overall, the colon of the DMH-induced rats demonstrated moderate–strong staining, and the IRS of the induced rat colon were 9, 5, and 4 for 9, 11, and 13 weeks, respectively, and showing different levels of inflammation among the

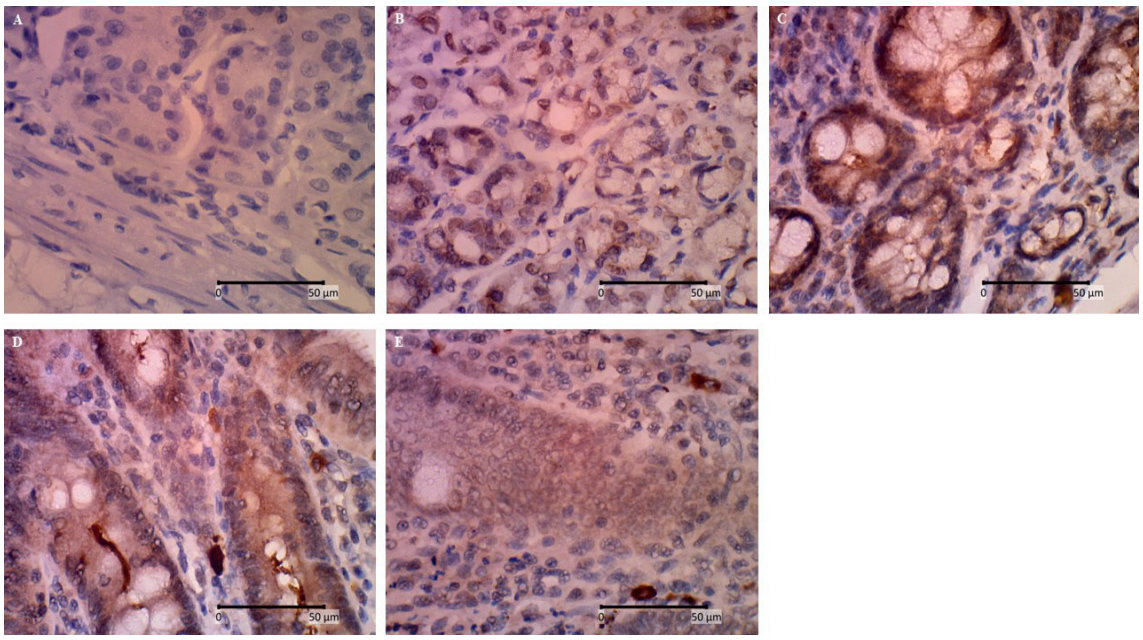

Figure 4. *COX-2* Expression. (A) Negative control staining was performed in the colon section of the untreated rats without antibody anti-COX-2. The negative control showed none of the immunopositive cells with a score of 0. (B) Positive control staining was performed in the colon section of the untreated rats with antibody anti-COX-2. The positive control showed immunopositive brown staining of the cytoplasmic cells with a score of 12. (C) Strong immunopositive staining in the colon of a week 9 DMH-induced rat. (D) Moderate immunopositive staining in the colon of a week 11 DMH-induced rat. (E) Mild immunopositive staining in a week 13 DMH-induced rat. All figures were captured at 400X magnification. Scale bar: 50 µm.

groups ($p < 0.0044$) (Table 4). Spearman's rank correlation coefficient test was performed between COX-2 expression and the severity of inflammation, and the results showed a very strong positive correlation ($R = 0.867$) (Table 5).

Discussion

DMH-induced rats represent a model of sporadic CRC. Several studies have shown that sporadic CRC in humans is a result of exposure to alkylating agents, such as DMH. This induction mainly harbors mutations in the β-catenin gene CTNNB1, which is also observed in hereditary nonpolyposis CRC. The mutation causes the β-catenin protein become resistant to regulatory degradation; thus, if β-catenin becomes stable and WNT signaling is increased, tumorigenesis occurs [21, 22]. DMH causes DNA methylation in various organs, including epithelial cells in the crypt, particularly in the proliferative area, which causes apoptosis of colonic cells and promotes the proliferation and mutation of epithelial cells in the colon [21].

The progression of adenoma to adenocarcinoma is a multistep process in DMH-induced carcinogenesis. As shown by histological changes, initiated by intraepithelial neoplasia or ACF, the proliferative zone in colonic crypts grew [21]. ACF are the earliest histological changes occurring during the multistep development of colorectal neoplasia and are classified into hyperplastic and dysplastic. Hyperplastic ACF can be seen as an elongation and enlargement of the crypt, whereas dysplastic ACF is also called microadenoma, which is characterized by neoplastic epithelial changes similar to those seen in tubular adenomas. Hereafter, the initial changes are called ACF, and the hyperplastic ACF is called hyperplasia [21, 23, 24]. Hyperplastic epithelial lesions contain both goblet and absorptive cells, with enlarged or occasionally crowded nuclei without stratification. The luminal opening of the crypts is slightly elevated compared with the surrounding normal mucosa, and the crypts are elongated with occasional branching and partial mucin depletion [21]. The lesions progress to adenoma, showing potential to progress further to adenocarcinoma [21, 23, 24].

This study showed that rats exposed to DMH at a dose of 30 mg/kg BW for 9, 11, and 13 weeks without a latency period still exhibited a normal colonic wall morphology with well-differentiated epithelium. ACF as an initial intraepithelial lesion can be seen in the week 11 group. In addition to ACF, hyperplastic ACF can also be seen in the week 13 group; however, we did not find any adenoma or adenocarcinoma in this study. Perse and Cerar [21] stated that ACF exhibited variable features from mild atypia to severe dysplasia, and most ACF showed a hyperplastic profile, whereas only a small group of ACF was found to cause intraepithelial neoplasms such as severe dysplasia, microadenoma, or carcinoma in situ [21]. Karaca et al. [10] mentioned that subcutaneous injection of DMH can cause 100% epithelial dysplasia and precancerous lesions in a 12-week study [10]. At the start of ACF formation, a nondysplastic stage is observed, consisting of non-hyperplastic and hyperplastic ACF. Non-hyperplastic ACF are characterized by enlarged crypts that are at least 1.5 times larger than normal, with no significant abnormalities in epithelial cells lining the crypts. Although minimally enlarged and elongated nuclei may be present, no crowding, stratification, or mucin depletion was observed [21].

ACF formation varied in terms of the duration reported previously, ranging from 4 to 12 weeks post DMH or AOM induction, with ACF consisting of one or two crypts [9, 25]. Rodrigues et al. [9] reported that 30 weeks after induction, approximately 46% of ACF had three or more crypts, and detected well-differentiated adenocarcinomas [9]. In a review article, Perse and Cerar [21] mentioned that ACF appeared 8–12 weeks after the injection of DMH or AOM, with tumors forming at week 40 [21]. In addition to the duration of induction, DMH doses ranged from 2 to 200 mg/kg BW with single to 30 injections with a latency period of 8 h to 78 weeks. The latency period is important for the development of colonic tumors [7]. In the present study, 9–13 weeks after DMH induction, without a latency period, only ACF formation was observed, and tumors had not yet developed. Tumor or adenocarcinoma formation requires a waiting period of 20–40 weeks, depending on the dosage and frequency of DMH administration [9, 21, 25–27]. The multistep development of CRC starts with non-dysplastic ACF, followed by subtypes of non-hyperplastic and hyperplastic ACF, dysplastic ACF, intraepithelial mucin-depleted foci, tumor, adenoma, and eventually adenocarcinoma [21, 28].

Other than epithelial changes in the colonic wall, inflammation in the colorectal mucosa can be caused by chemical agents that induce a chronic immune response to promote cell proliferation and regeneration [29] and this is one of the characteristics of early lesions in the pathogenesis DMH-induced CRC [7]. When injury is not healed, the microenvironment will become enriched by various factors, including cytokines and growth factors, to prolong proliferation to repair tissues. Nevertheless, instead of being repaired, genetic errors accumulated, which progressed to impaired proliferation [29].

In this study, at week 9 of DMH induction, severe inflammation that represents acute inflammation occurred. Acute inflammation is the initial reaction to harmful triggers and typically lasts for a few days or weeks. During acute inflammation, granulocytes account for most of the infiltrating cells, and acute inflammatory cytokines, such as IL-10, IL-12, and IFN-γ, are produced to inhibit the progression of tumors. However, when proinflammatory cytokines persist beyond the acute inflammation phase, chronic inflammation occurs [30, 31].

In chronic inflammation sites, where the development of tumors is initiated, M1 macrophages are the predominant macrophage population. M1 macrophages produce large amounts of proinflammatory cytokines, such as TNF-α, IL-12, reactive nitrogen/inducible nitric oxide synthase and oxygen intermediates/ROS. The activated macrophages produce IL-23, which will then trigger IL-17 responses from resident macrophages and induce stromal, epithelial, and endothelial cells and some subsets of monocytes to produce IL-1, IL-6, IL-8, and TNF-α. These inflammatory cytokines will recruit neutrophils

to the inflammation site. When tumor invasion begins, macrophages evolve into their M2-like phenotype [29].

In this study, the severity of inflammation from weeks 9 to 13 week gradually decreases from moderate–strong (week 9), moderate (week 11), and then low–moderate (week 13), which was accompanied by a shift in the levels of *COX-2* from strong (week 9) to moderate (weeks 11 and 13), respectively (Tables 3 and 4). A very strong positive correlation (R = 0.867) was found between the severity of inflammation and *COX-2* expression.

COX-2 is the key regulator of inflammation, and its expression increased in several cancers. COX-2 overexpression is associated with inflammation, resistance to apoptosis, uncontrolled proliferation and growth, neovascularization, angiogenesis, and finally cancer [32]. COX-2 is primarily expressed in inflammatory cells and significantly upregulated during chronic and acute inflammations. In carcinogenesis, COX-2 expression levels can be consistently high. Several cytokines such as IL-1β and TNF-α can stimulate the expression of COX-2. COX-2 catalyzes the production of prostaglandin E2 (PGE2) in colorectal fibroblasts [13, 30, 33]. COX-2 induces prostaglandin generation, including PGE2 that serves as an immune suppressor. It suppresses the macrophage- and natural killer cell-mediated cytotoxicity [32]. COX-2 overexpression has been observed in premalignant and malignant stages [5].

COX-2 is activated in response to many intracellular and extracellular stimuli, including TNF-α [32]. This study showed diffused high-intensity expression of TNF-α in all groups. Although the expression levels of COX-2 at weeks 11 and 13 of DMH induction were lower than those at week 9, it remained at a moderate level. This finding is consistent with the aforementioned statement that TNF-α can stimulate COX-2 expression.

TNF-α initiates chronic inflammation and is frequently present in the tumor microenvironment [34]. TNF-α-induced inflammation is an important factor in CRC initiation and establishment. TNF-α induces the early phase of inflammation and controls the production of various cytokines and other processes of tumor development, such as increasing vascular permeability and oncogene activation, angiogenesis, and tumor invasion [5, 30, 31, 35]. TNF-α plays an important role in the initiation and perpetuation of colon cancer [36, 37].

This study only examined the duration of induction. To follow the morphological changes of the colonic wall from the initiation of tumorigenesis until the development of adenocarcinoma, a latency period is required. In addition to the tested parameters, other crucial parameters that are involved in the pathogenesis of CRC must be evaluated; thus, the morphological changes can be confirmed with the ongoing stage of CRC tumorigenesis.

In conclusion, the DMH induction in Sprague–Dawley rats at 30 mg/kg BW for 9, 11, and 13 weeks without a latency period is not enough to induce adenoma and adenocarcinoma. All the induced rats showed normal colonic walls with inflammation infiltrates that can be seen in between the epithelial cells and lamina propria. The degree of inflammation among the groups was different. Non-hyperplastic and hyperplastic ACF were observed, and the expression levels of TNF-α and COX-2 were maintained among the groups. To understand further the inflammation process in CRC carcinogenesis, several other parameters must be further explored.

Author Contribution Statement

Albertus Ari Adrianto : design of the work, data acquisition, data analysis, drafting manuscript, final approval of the published data. Ignatius Riwanto : design of the work, revising the intellectual content. Udadi Sadhana : revising the intellectual content, data interpretation. Henry Setyawan : revising the intellectual content, data analysis. Endang Mahati : revising the intellectual content. Sitarina Widyarini : data interpretation, revising the intellectual content. Afranetta Aulya Asri Wandita : data acquisition, data interpretation, data analysis, drafting manuscript. Dewi Kartikawati Paramita : revising the intellectual content, data analysis, drafting manuscript, final approval of the published data.

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General

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Ethical Declaration

This study is approved by the Animal Ethics Committee of Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia with the ethical committee approval number "No.93/EC/H/FK-UNDIP/IX/2020."

Data Availability

The data that support the findings of this study are available from the corresponding author, DKP, upon reasonable request. When you need to access the data, please ask to the corresponding author by clicking the link https://docs.google.com/spreadsheets/d/1Rg6zq8LdfPgnp0f8iljjFTnhZRKFH0Sk/edit?usp=drive_link&ouid=112657006827135210916&rtpof=true&sd=true, and request access.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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