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Review Article

REVIEW ARTICLE

Biochemical and molecular aspects of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis: a review

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Abstract

1,2-dimethylhydrazine (DMH) is a member in the class of hydrazines, strong DNA alkylating agent, naturally present in cycads. DMH is widely used as a carcinogen to induce colon cancer in animal models. Exploration of DMH-induced colon carcinogenesis in rodent models provides the knowledge to perceive the biochemical, molecular, and histological mechanisms of different stages of colon carcinogenesis. The procarcinogen DMH, after a series of metabolic reactions, finally reaches the colon, there produces the ultimate carcinogen and reactive oxygen species (ROS), which further alkylate the DNA and initiate the development of colon carcinogenesis. The preneolpastic lesions and histopathological observations of DMH-induced colon tumors may provide typical understanding about the disease in rodents and humans. In addition, this review discusses about the action of biotransformation and antioxidant enzymes involved in DMH intoxication. This understanding is essential to accurately identify and interpret alterations that occur in the colonic mucosa when evaluating natural or pharmacological compounds in DMH-induced animal colon carcinogenesis.

Key words: colon cancer; DMH; preneoplastic lesions; chemoprevention

Background

Colon cancer is the third most leading cancer in males and the second most leading cancer in females in the industrialized countries, and its morbidity and mortality are increasing in the developing countries [1]. Previously, the incidence of colon cancer was low in India and underdeveloped countries, but later studies showed a drastic increase in the colon cancer incidence in Asia [2]. Dietary habits play a crucial role in the development of colorectal cancer. Rapid urbanization and extensive growth of economic conditions influence the people to adopt western dietary style, which consists of high-fat, high-protein, low-carbohydrate, and low dietary fiber. This unbalanced diet is considered to be an important causative factor for the increased mortality in recent years [3]. In olden days, people consumed natural substances

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with lots of medicinal properties, therefore, they all away from these kind of diseases.

Influence of age, body weight, and sex

The population-based cancer incidence report shows that the morbidity has increased in the past century. The high rate of cancer incidence due to two major factors (i) our life expectancy is more (ii) exposure of cancer-causing chemicals and radiations, X-ray and plane travel and other sources. Today we live 30 years longer than last century. Moreover, cancer is common in older tissues, and elders are more susceptible than youngsters. The laboratory and clinical studies revealed that cancer is an ancient disease; it is not one disease, more than 100 diseases.

Age. The risk of developing colorectal cancer increases with age. Colon cancer is most common in over 50 years of aged people, and a chance of getting colon cancer is higher in each decade. However now the incidence is increasing in younger people also.

Body weight. Obesity is one of the risk factors of colon cancer. In particular, when comparing with healthy men, overweight men (BMI $>$ 30 kg/m^2) have a higher risk of 53%. Sedentary lifestyle may be responsible for 13–14% of colon cancer incidence. It is an attributable risk greater than family history.

Sex. Male rats are most frequently used to study preneoplastic and neoplastic lesions in colon carcinogenesis, due to their increased susceptibility to colon carcinogens [4].

Incidence

The incidence of colon cancer is high in male compared to females. Hormonal factors may play a role in having less percentage of colorectal cancer in females. Microsatellite instability is one of the molecular changes in colon cancer. A case-control study examining sex, reproductive factors, and hormone exposure related with microsatellite instability in colon cancer suggested that estrogen exposure is a protective factor against microsatellite instability, while the lack of estrogen in older women increased the risk of microsatellite instability—high risk of colon cancer [5]. Nevertheless, the researchers studied the efficiency of DMH in female mice and found 83% of mice developed visible tumors and many possess especially in the distal part of the colon [6, 7]. The action of DMH induction in mice shows enlargement of proliferative zone in the colonic crypts, which led to an increment in the total number of labeled cells in the crypts and decrement of the activity of enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) (found in human colonic neoplasm).

Changes in the bowel habits also one of the causative factors for colon cancer [8], which includes (i) increase in intestinal transit time (ii) tiny stool, which consequences of thickening of luminal content (iii) possible interactions with various carcinogens or promoting factors present in the intestine, thus hard stools and constipation might be expected to enhance carcinogen exposure.

1,2-Dimethylhydrazine

Role and history of DMH

Dimethylhydrazine occurs as 1,2-dimethylhydrazine and 1,1-dimethylhydrazine isomers. Both compounds are clear, colorless liquids [9]. 1,1-Dimethylhydrazine is used in the rocket and jet as fuel and is also used as a growth control agent in plants and as a feedstock in chemical syntheses.

DMH and its metabolite, azoxymethane (AOM), are procarcinogens that require metabolic activation to form DNA-reactive products. The alkylating agents DMH and AOM begin their mutagenic activity through the methylation of guanine in DNA at N-7 position. The alkylated guanine is paired with thymidine instead of cytosine by donating a proton, which leads to the modification of bases. Further subsequent replication, mispairing of guanine to thymine and cytosine to adenine, occurs, which leads to mutations in DNA. Metabolism of these procarcinogenic compounds involves various metabolic enzymes, including xenobiotic-metabolizing enzymes, these enzymes process several N oxidation hydroxylation stages, including the formation of ultimate carcinogen methylazoxymethanol (MAM). MAM is a reactive metabolite of DMH and AOM, which readily yields methyldiazonium ion that can alkylate macromolecules in the liver and colon [10], proved by various studies [11, 12].

MAM is a substrate of the nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase present in the colon and liver, suggesting that the active metabolite of MAM might be the corresponding aldehyde [13, 14], and these metabolites of CYP2E1 are transported to the colon via the bile or bloodstream. The main pathway involves the hepatic conversion of DMH to AOM and azoxymethanol which subsequently undergoes glucuronic acid conjugation and biliary excretion [15]; however, the toxicity of azoxymethanol doses affects the liver, cell membranes, and other organelles, which is supported by the release of aspartate and alanine amino transferases and alkaline phosphatase [16, 17]. The glucuronides reaches the colon, and it further undergoes hydrolysis by bacterial enzymes to produce active carcinogen in the colonic lumen Fig. 1 [18].

The history of DMH starts around 1965, Laquer (1965) investigating the neurotoxicity for seed of *Cycas circinalis L*. Rats fed with crude extract of cycad meal produce tumors in various organs including the intestine, liver, and kidney. Further he found that a glycoside, cycasin, and a β-D-glucosyloxyazoxymethane isolated from the crude material and the first metabolite of aglycone cycasin (MAM) are responsible for the tumors in the intestinal tract [19].

Initially Fiala [20] investigated the metabolism and mode of action of DMH, analyzing its metabolites, and separated them by column chromatography. Further he conducted a study with DMH and AOM [15], which confirms that the active carcinogen is not transported through fecal stream rather circulatory system, which was proved by Campbell *et al.* and Wittig and Ziebarth [21, 22].

Routes and dosage of DMH administration to rodents

The main route of administration of DMH is subcutaneous injection [23]. Even though it is an effective method to induce tumor, intraperitoneal injections also succeeded to produce tumors in the colon [24], whereas single injection intrarectal exposure of DMH also produced tumors in the colon of germ-free mice [25, 26].

The s.c. injection of DMH causes 100% epithelial dysplasia and precancerous lesions, found in a 12-week study [27]. DMH causes a wide range of tumors in the GI tract of C57BL/6 mice, and the majority of tumors found in small intestine and colon in the respective studies [28, 29]. Even though the majority of experimental colon cancer study carried out in rats, the high frequency of tumor in lower part of colon, a histopathological

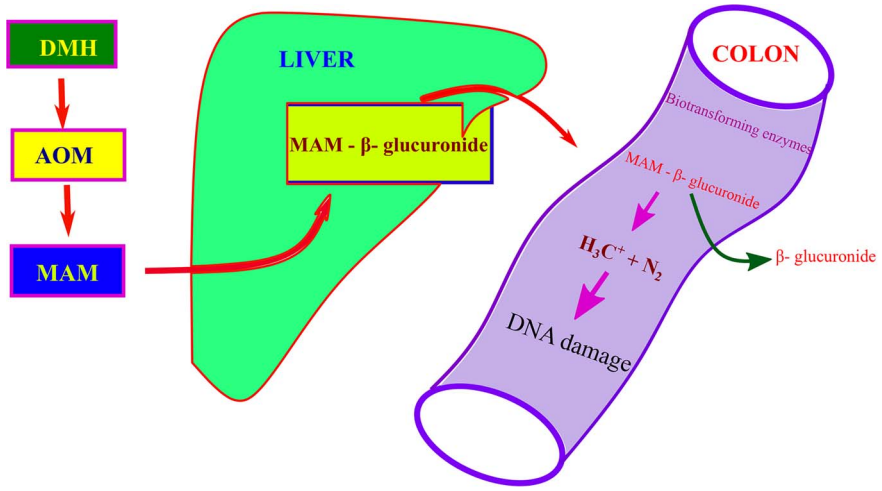

Figure 1: Transport of DMH from subcutaneous site to colon through glucuronidation.

evidence of multiple adenomas and subsequent progression of adenocarcinoma also validates the importance of mice in pathogenesis of colon cancer (Table 1) [30, 31].

Various doses of DMH can produce the colonic tumors. The dosage ranging from 2 mg to 200 mg/kg b.w. (single injection to 30 injections); and the duration ranging from 8 hours to maximum 78 weeks of latency period (which important for the development of colon tumor) (Table 2), can induce colon tumors in DMH studies regardless of the route of the administration. In the recent years, the dosage levels and routes of administrations were standardized, depending on their experimental studies, 15 mg and 20 mg/kg b.w [32]. DMH and AOM at the dosage levels of 10–20 mg/kg b.w. produce adenomas and adenocarcinomas in rodent model [33]. Even a single injection of 10 mg/kg DMH or AOM produces colon cancers in rats after a latency period of 15–20 months Fig. 2 [34].

Metabolism of DMH

An understanding about the chemical carcinogen and mechanism of action is necessary to develop procedures for diagnosis and eventually prevention of the disease.

A number of hydrazine derivatives are found in the environment, industry, agriculture, and medicine [35]. The organotropic colon carcinogen DMH is an alkylating agent widely used to induce benign and malignant neoplasm in the colon of rodents [36].

In animal studies, repeated exposure of DMH produces colon tumors, which shows the pathological features that are similar to sporadic forms of human colon cancer [37]. In most of the studies, carcinogen DMH was injected subcutaneously. The subcutaneous site of injections does not possess the enzymes able to metabolize or react with DMH. Hence, subcutaneously administered DMH is released into the circulation slowly, and then it reaches the liver and gets metabolized into various intermediates [15]. Metabolic activation of DMH is shown in Fig. 3.

The first oxidation step of DMH involves its oxidation to azomethane, which is converted into azoxymethane (AOM) and then hydroxylated to methylazoxymethanol (MAM). Hydroxylation occurs predominantly in the liver, probably via cytochrome P450-dependent pathway and to a limited degree in the colonic mucosa [18]. MAM can reach the intestine through bile, intestinal lumen directly and also via circulation [18], as glucuronides and glucosides to some extent as sulfates. They are cleaved by β-glucuronidase, β-glucosidase, and sulfatase enzymes which are present both in the enterocytes and also in the intestinal microflora. MAM is chemically unstable at body temperature and decomposes spontaneously to formaldehyde, water, and nitrogen [38]. During this decomposition, the alkylating agent methyldiazonium ion is formed, which generates a reactive carbonium ion capable of alkylating macromolecules (DNA, RNA, or protein) by enzymic and non-enzymic process in the colon. Alkylation of the oxygen atoms present in nitrogenous bases turns the possibility of mispairing of DNA, which has been suggested to be critical in mutagenesis and carcinogenesis [39]. Alternatively MAM has been found to be a substrate for NAD^+-dependent dehydrogenase of the colon and liver, suggesting that the active metabolite of MAM may be the corresponding aldehyde Fig. 3 Metabolism of DMH [40].

Diversity in species

The carcinogenic effects of DMH in different animal species and strains and their target organs are listed in Table 2.

The DMH-induced colon tumors in hamsters are sparse [41, 42]. Both studies evaluated the pathological analyses of colon tumors in hamsters; the percentage of tumor occurrence and severity varies slightly due to the dosage and routes of administration. However, the pathogenicity and metastasis are high in carcinogen injected in s.c. rather than other routes [42, 43]. However, on contradictory, few studies showed that DMH has no tumorigenic influence in Syrian golden hamsters, which shows the species specificity and route specificity of DMH [44–46]. Apart from that, DMH administered to hamster through drinking water was reported to induce cancer in various organs [43] and [45, 46] causes hepatic lesions, forestomach papillomas, and adenocarcinomas of the colon [47]. Even the DMH has the ability to produce tumors in monkey's colon; many researchers believe that monkeys are resistant to effects of chemical carcinogen for colon since tumors arise in different organs [48, 49].

Promoters

Dietary fat appears to act as a promoter rather than an initiator of colon tumor. Dietary fat may provide a favorable environment for the development and growth of tumor cells, by altering the

Table 1: List of some effective chemopreventive agents against DMH-induced colon cancer

S. No	Chemopreventive agent	Dosage level (mg/kg.b.w)	Species and strain		Biomarkers studied	Outcome	Reference
1.	Olive oil	1000	Male Sprague Dawley rats	↓ ↑	mRNA expression of NF-κB, VEGF, MMP mRNA expression of caspases 3 and 9	Chemoprevention	[144]
2.	p-Coumaric acid	100	Male Wistar rats	↓ ↑	Bcl-2, I$\alpha\beta\alpha$, cyt C, caspase-3, p65 P53, GSp78	Antiproliferative	[145]
3.	Rosmarinic acid	5	Male Wistar rats	↓ ↑	CYP450, CYP450 2E1, NADPH, CYP450 red, NADH-cytb5 red LOOH, CD. Phase II drug-metabolizing enzymes	Chemopreventive, proapoptotic	[8]
4.	Myrtenal	230	Male Wistar rats	↓ ↑	CYP450, Cytb5, UDPGT, GST SOD, GPx, GR, VIT C, VIT E	Anticarcinogenic, anti-inflammatory	[146]
5.	p-Methoxycinnamic acid	40	Male Wistar rats	↓ ↑	NF-κB, iNOS, COX-2, TNF-α, IL-6, cyclin D1, BCl-2 p53, Bax	Antiproliferative, anti-invasive	[147]
6.	Etoricoxib	6	Male Sprague Dawley rats	↓ ↑	Alkaline phosphatase, maltase, lactase, LPO SOD, CAT, GST, GSH, NO, citrulline	Chemopreventive, protects intestinal membrane	[148]
		0.64		↓	COX-2, NF-κB	Anti-inflammatory, anticancer	[149]
7.	Caraway seed oil (*Carum carvi* L)	0.0.1%	Male Wistar rats	↑	TBARS, GSH, SOD, CAT, GSH, CYP1A1	Chemopreventive, modulator drug-metabolizing enzymes	[150]
8.	Black cumin oil (*Nigelle Sativa* L)	200	F344 rats				
9.	Coriander seeds (*Coriandrum sativum*)	10% powdered coriander seeds	Sprague Dawley rats	↓ ↑	Cholesterol Phospholipids, Neutral salts, Bile acids	Antihyperlipedemic, membrane fluidity, and integrity	[57]
10.	*Amorphophallus campanulatus*	250	Male Wistar rats	↑	GSH, GST, GR, GPx, CAT	Chemopreventive, antiproliferative	[151]
11.	Bis-1,7-(2-hydroxyphenyl)-hepta-1,6-diene-3,5-dione (a curcumin analog)	80	Male Wistar rats	↑	GSH, GPx, GST, SOD, CAT	Chemopreventive, detoxification of carcinogen	[152]
12.	Silibinin	50	Male Wistar rats	↓ ↑	CYP450, CYP 450 2E1, b5 red, CYP450red, TBARS, LPO, CD GST, UDPGT, DTD, SOD, CAT, GPx	Chemopreventive, modulator phases I and II	[153] [53]
13.	Aloin	100/p.o.	Wistar rats	↓ ↑	MDA, TNF SOD, CAT, GSH, GPx, GR, GST	Inhibit tumor promotion, anti-inflammatory	[154]
14.	Ellagic acid	60/p.o.	Wistar rats	↓ ↑	LPO, pAKT, Bcl-2 SOD, CAT, GPx, GR, GST, GSH, Vit C and E, Total AKT, BAX, CytC, Cas 3	Chemopreventive, proapoptotic, inhibit carcinogen activation	[155, 156]
		40/p.o	Wistar rats	↓	Cathepsin D, ALP, LDH, ODC, c-myc		[157]
15.	Diclofenac	8/p.o.	Male Sprague Dawley rats	↓ ↑	COX-2, NF-κB PPARγ, Apaf	Chemopreventive, anti-inflammatory	[158]
16.	Ginger (*Z. officinale* Rosc)	50/p.o.	Wistar rats	↑	SOD, CAT, GPx, GST, GR, GSH, VIT A, E, C	Inhibiting during imitation stage, increase antioxidant	[159]

Continued

Table 1: Continued.

S. No	Chemopreventive agent	Dosage level (mg/kg.b.w)	Species and strain		Biomarkers studied	Outcome	Reference
17.	Capsaicin	50/p.o.	Wistar rats	↓	ALT, AST, Caspase-3	Reduced cell proliferation, chemopreventive	[160]
18.	Morin	50/p.o.	Male albino Wistar rats	↓	TNF-α, COX-2, IL-6, PGE-2, NF-κB,	Anti-inflammatory, proapoptotic	[161]
19.	Naringenin	50/p.o.	Male albino Wistar rats	↓	ROS, MDA, TNF-α, NF-κB, COX-2, iNOS	Chemopreventive, anti-inflammatory	[162]
20.	Orientin	10/i.p.	Male albino Wistar rats	↓	NF-κB, TNF-α, IL-6, iNOS, COX-2, mast cells	Chemopreventive, anti-inflammatory	[163]
21.	Green tea	1% (W/V) mixed with drinking water	Male albino Wistar rats	↓	NF-κB, TNF-α, iNOS, COX-2, VEGF, MMP	Anti-inflammatory, anti-angiogenic	[164]
22.	*Copaifera langsdorffii*	40/p.o.	Male albino Wistar rats	↓	Comet, ACF	Chemopreventive	[165]
23.	β-Sitosterol	20/p.o.	Male albino Wistar rats	↓	ACF, crypt multiplicity	Chemopreventive	[166]
24.	6-Methylsulfinyl hexyl isothiocyanate	40/p.o.	F344 rats	↓	ACF, BCAC, PCNA	Chemopreventive, antiproliferative	[167]

composition of cell membranes. Epidemiological studies have shown that high-fat diet consumption leads to elevated fecal concentrations of secondary bile acids in the colonic lumen, which stimulate hyperproliferation and thus increase the incidence of colon cancer [50]. High-fat diet, rich in ω-6 polyunsaturated fatty acids, fed in animals during the carcinogenic stage, increases the incidence of colon tumor. It is possible that the high incidence of colon tumors in rats fed with a high-fat diet along with DMH observed in few studies [51–54] may be due to the excretion of elevated amounts of bile acids, which act as colon tumor promoters [55]. High levels of bile acids associated with the high-fat diet can initiate membrane stress signaling, leading to the activation of several complex pathways and alteration in membrane components like cholesterol, thereby disrupting cell membrane integrity. Alternatively, it has been proposed that bile acids increase the risk of colon cancer by inhibiting phase I and phase II xenobiotic-metabolizing enzyme systems located in hepatic and extrahepatic tissues including the colon [50].

High-fat diet and DMH, however, significantly increased HMG CoA reductase activity and cholesterol synthesis in the liver. The enhanced HMG CoA reductase activity may contribute to the modulation of cell growth, cell cycle arrest, inhibition of apoptosis, and cellular signaling activities in DMH-exposed animal studies [56].

Apart from that, DMH exposure increases the cholesterol accumulation, which was benefitted by tumor cells. Cooper *et al.* have observed that DMH treatment results in doubling of biological membrane cholesterol and gross distortion of cell shape, with changes in the (i) lipid composition, (ii) membrane fluidity, and (iii) morphology [36]. This in turn may present the accumulation and metabolism of secondary bile acids by the colon microflora [57]. The enhancing effect of colon tumor by the high-fat diet depends on the type of fat and their fatty acid composition. In general, dietary fat that contains linoleic acid, a precursor of prostaglandin, which is effective in promoting tumorigenesis in animals [58].

During the progression stage, weight loss is the common feature of the colon cancer, which reflects the aggressiveness of the disease. The tumor burden in the colon results in decreased food intake, therefore decreasing weight gain or increasing weight loss. Alterations in the glucose metabolism and elevated hepatic gluconeogenesis deplete the energy sources, all together results in significant weight loss [59]. The cachexia (catabolic clinical state) may be caused by a combination of endocrine and immunological disturbances resulting from host-tumor interactions. Different mechanisms are involved in the weight loss of starvation and cancerous condition. During the starvation, the weight loss is mainly from adipose tissue and small amount from tissues, whereas in cancer loss, it will be in both adipose tissue and skeletal muscles. In starvation, ketone bodies are produced from fat, which replace glucose, therefore inhibiting the loss of muscle mass.

Preneoplastic Lesions

Observations of preneoplastic lesions

The preneoplastic lesions, which were phenotypically altered by carcinogens, but still they lack of important properties of final tumor cells. Aberrant crypt foci (ACF) initially were identified topographically as the earliest recognizable lesions on the carcinogen-exposed rodent colons. ACF were first described by Bird [60] in methylene blue-stained whole-mount preparations of rodent colon, treated with colon-specific carcinogens.

Table 2: The carcinogenic effects of DMH in different animal species and strains and their target organs

S.No	Dose/route	No. of injections	Total duration of the experiment (weeks)	Affected organs	Tumor arised	Metastasis	Strain	References
1.	10 mg/kg b.w./s.c.		24	Colon	Large and small intestines		Male Wistar rats	[168]
2.	20 mg/kg b.w./s.c.		24	Colon, liver, small intestine	Large and small intestines	Liver	Male Wistar rats	[168]
3.	35 mg/kg b.w/Oral		78	Small intestine, Zymbal gland			Male Fischer rats	[169]
4.	30 mg/kg b.w./s.c.		20	Colon	Colon		Male Wistar	[170]
5.	30 mg/kg b.w./i.p.	12	24	Small intestine. Colon	Colon	Small intestine	Male Swiss mice	[171]
6.	40 mg/kg b.w./s.c.	4	5	Colon			Male Wistar rats	[165]
7.	30 mg/kg b.w./s.c.	30	30	Colon	Colon		Male Wistar rats	[164]
8.	30 mg/kg b.w./s.c.	16	16	Colon	Colon		Male Wistar rats	[172]
9.	75 mg/kg b.w./s.c.	1	24 hours	Colon			Male F344 rats	[173]
10.	21 mg/kg b.w./s.c.	12	28	Colon			C57 black male mice	[174]
11	21 mg/kg b.w.	15	26	Colon	Colon	Duodenum, jejunum	Female LIO rats	[175]
12.	40 mg/kg b.w./s.c.	4	4	Colon			Male Wistar rats	[176]
13.	40 mg/kg b.w./i.p.	10	10	Colon	Colon		Male Wistar rats	[177]
14.	40 mg/kg b.w./s.c.	8	8	Colon	Colon		Male F344 rats	[178]
15.	40 mg/kg b.w./s.c.	2	2	Colon	Colon		Male Wistar rats	[179]
16.	200 mg/kg b.w.	1	2 days	Liver	Not measured		Fischer 344 rats	[180]
17.	80 mg/kg b.w./i.p.	1	8 hours	Colon			Male BDF1 mice	[181]
18.	15 mg/kg b.w./s.c.	20	52	Colon	Colon		Male F344 rats	[182]
19.	50 mg/kg b.w./s.c.	10	35	Colon, liver, small intestine	Colon	Caecum, small Intestine, liver, abdomen	Male Wistar rats	[183]
20.	30 mg/kg b.w./s.c.	10	26	Colon	Colon		Male F344 rats	[184]
21.	25 mg/kg b.w./s.c.	1	7 days	Liver			Male Wistar rats	[185]
22.	125 mg/kg b.w./i.p.	1	14 days	Colon			Male Wistar rats	[186]
23.	20 mg/kg. b.w./s.c.	15	30	Colon	Colon		Male Wistar rats	[163]
24	20 mg/kg. b.w./s.c.	8	31	Colon	Colon		F344 rats	[187]
25.	20 mg/kg. b.w./s.c.	15	30	Colon	Colon	Caecum	Male Wistar rats	[62]

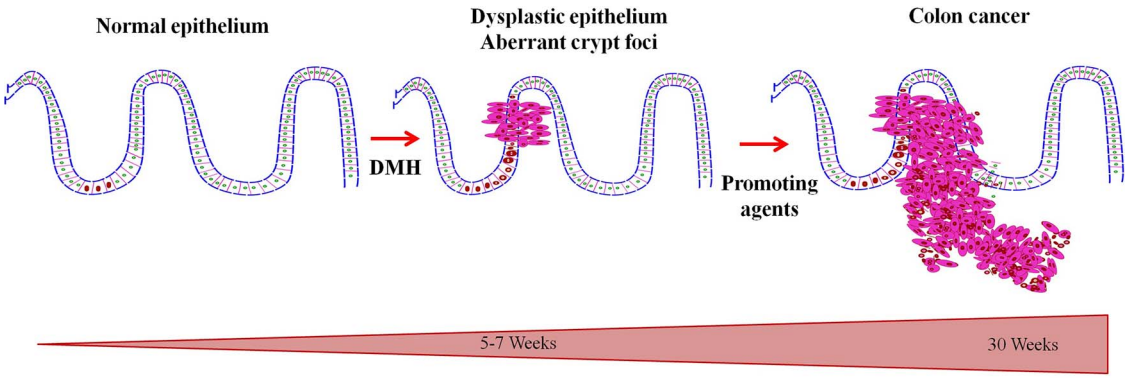

Figure 2: DMH injections induced the mutation in normal epithelium, later multi-step conversion of mutated epithelium to malignant one by 30 weeks.

McLellan and Bird defined aberrant crypts to have the following structural features: (i) they are larger than the normal crypts in the field, (ii) have elevated pericryptal zone that separates them from the normal crypts, (iii) have a thicker layer of epithelial cells that often stains darker, and (iv) generally have oval rather than circular openings (normal crypts) [61]. They can be observed as single altered crypts or as a group of altered crypts that appears to form a single unit or focus. Frequently, ACF are microscopically elevated above the mucosa but also may be depressed, i.e. usually they are not in the same focal plane as the surrounding normal crypts. Crypts harboring any four of the abovementioned criteria are considered as aberrant crypts.

DMH treatment induces the formation and multiplicity of ACF, which reflects the initiation stage of colon cancer in rats. Alternatively, high-fat diet also increased the multiplicity of ACF due to its tumor-promoting capabilities. In addition to increased number of ACF, luminal alterations, goblet cell reduction, and nuclear alterations of the cells surrounding the lumen of the crypt are also found [62]. These characteristics can be correlated with outcome of dysplastic ACF. Several studies have suggested that the growth features of ACF and dysplastic ACF and their location are used as a measure of the biological efficacy of the modifiers of colon carcinogenesis [31, 63]. Moreover, ACF and dysplastic ACF represent the earliest detectable lesions (preneoplastic lesions) in the development of colon cancer [64].

Apart from this, western diet alone could produce neoplasm in the colon of experimental animals. The large intake of high-fat diet along with low levels of intake of calcium, vitamin D, folate, choline, methionine, and fiber can induce adenomas and carcinomas of C57BL/6 mice without the exposure of carcinogen [29, 65], by depletion of apoptosis changes in cell renewal showed morphologically identifiable atypical mitotic figure (Fig. 4) [65].

Beta-catenin-accumulated crypts

Venkatachalam *et al.* demonstrated that the β-catenin-accumulated crypts in the sectioned colonic tissues exhibited histological abnormalities, including the disruption of cellular morphology [62]. Moreover, the β-catenin accumulation in the crypts increased with time after the carcinogen treatment. The crypts with the accumulation of β-catenin were infrequently recognized as adenomatous crypts having extensive branching. Previously, Yamada *et al.* reported that β-catenin-accumulated crypts in the large bowel harbor frequent mutations in the *β-catenin* gene, providing evidence that those crypts are premalignant lesions [66].

Histopathology of DMH-Induced Colon Tumors

The evaluation of the changing patterns of tumor development plays a valuable role in the development of anticancer agents. A mass of small adenomas viewable in colon carcinogen exposed rats, which were attached directly to colon and deeply stained red surrounding mucosa and becomes pedunculated. The carcinogen exposure brings severe abnormalities in adenomas (like architecture, cytology, and differentiation) which are expressed as dysplasia. This can be expected as multiple changes in the metabolic pathways that occur on DMH treatment during the conversion of adenomas to adenocarcinoma including growth factors promoting stromal proliferation and angiogenesis, proteolytic enzymes facilitating local invasion, numerous changes in the secretory and membrane-associated glycoproteins, alterations in cell adhesion molecules, and the development of aneuploidy [67]. The drastic increase in the total number of tumors in DMH-exposed rats revealed the rapid conversion of adenoma to adenocarcinoma and the growth of colonic tumors.

The earliest microadenoma is the unicryptal adenoma. The unicryptal adenoma starts as a little outgrowth (a bud) from the side of an apparently normal crypt [68, 69]. This little growth forms a tubule that moves upwards with the normal migration of the crypt column until it reaches the surface epithelium. The neoplastic tubule is usually shorter than its normal counterpart but undergoes fission to produce an oligocryptal adenoma. Growth that is expansile leads to the formation of a polyp [70]. Those adenomas are often flat or depressed but may subsequently become polypoid as their size increases [71].

The last stage of conversion of adenoma to adenocarcinoma must be a rate-limiting step, since adenomas are relatively large in numbers when comparing carcinomas. Furthermore, this step is accompanied by a multiplicity of phenotypic changes implicating enzymes in metabolic pathways [72], increased telomerase activity [73], growth factors promoting stromal proliferation and angiogenesis [74–76], proteolytic enzymes facilitating local invasion [77–80], numerous changes to secretory and membrane-associated glycoproteins [81], alterations in cell adhesion molecules [82], as well as the development of aneuploidy [83].

Role of Bacterial Enzymes

The greatest number of bacterial cells is found in the digestive tract of the human body. Fundamental comparative studies of human fecal microbiota have revealed the astonishing fact that each of us has unique microbiota (i.e. there are considerable

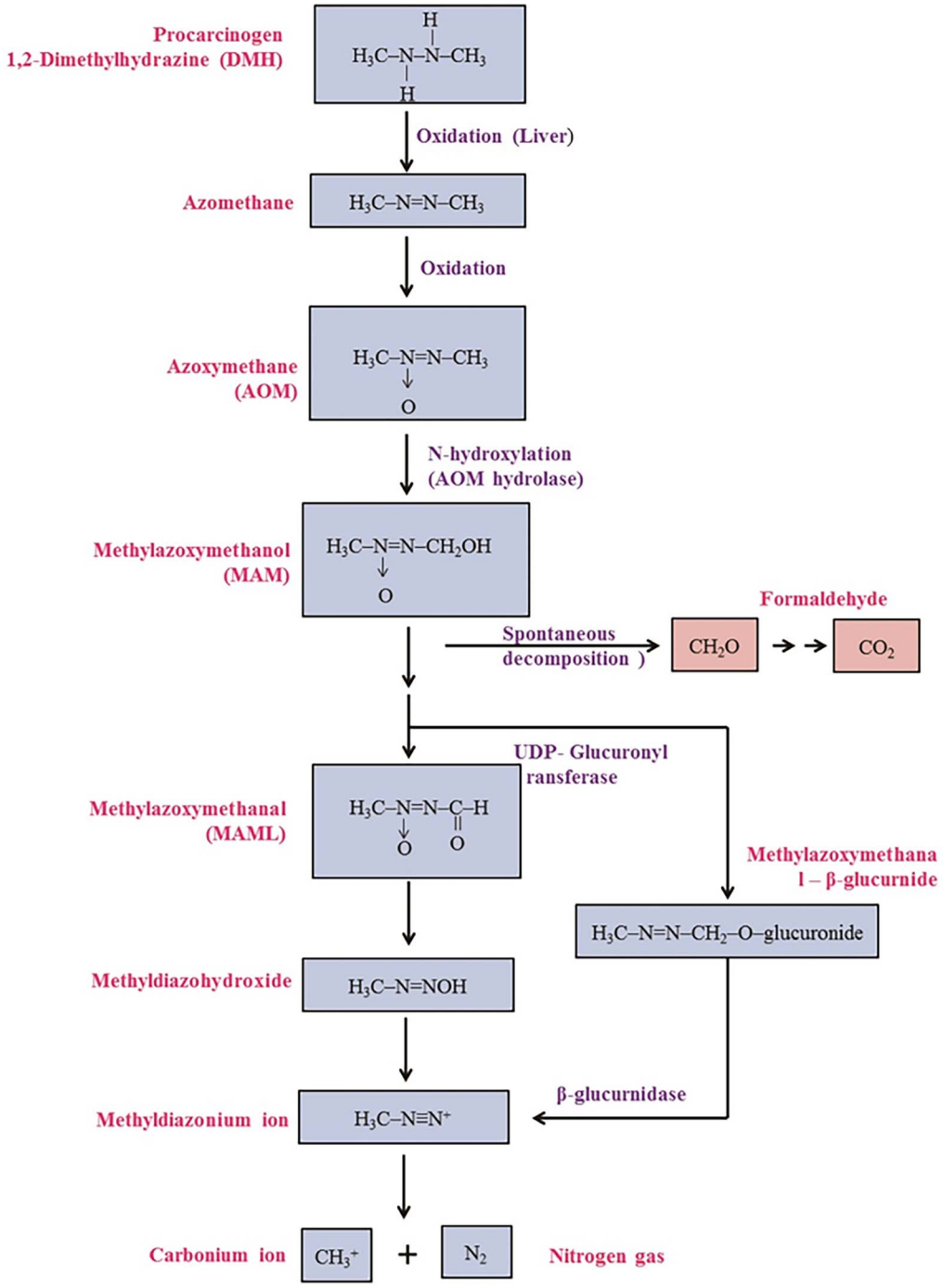

Figure 3: Metabolism of DMH.

differences between the compositions of the microbiota of individuals).

Experimental and clinical studies suggest that there is a link between consumption of red meat, high fats, and low vegetable intake, and alterations in the composition of the gut microbiota have been observed in animal and human studies. This high-fat diet has an impact on increased activities of some fecal bacterial enzymes, as well as modification of sulfidogenesis and biliary acid metabolism with an impact on development of procarcinogenic conditions [84].

Intestinal bacterial enzymes such as β-glucuronidase and β-glucosidase release toxic metabolites from nontoxic glycoside conjugates and extend the exposure time of the toxicant in the body. β-glucuronidase is responsible for the conversion of glucuronide conjugate in the lumen of the gut [85], which leads to the generation of toxic (or) carcinogenic compounds by cleaving the terminal glucuronic acid which was earlier detoxified in the liver by glucuronidation. High-fat diet along with DMH exposure shoots up the fecal β-glucuronidase activities [51]. The increased activities may

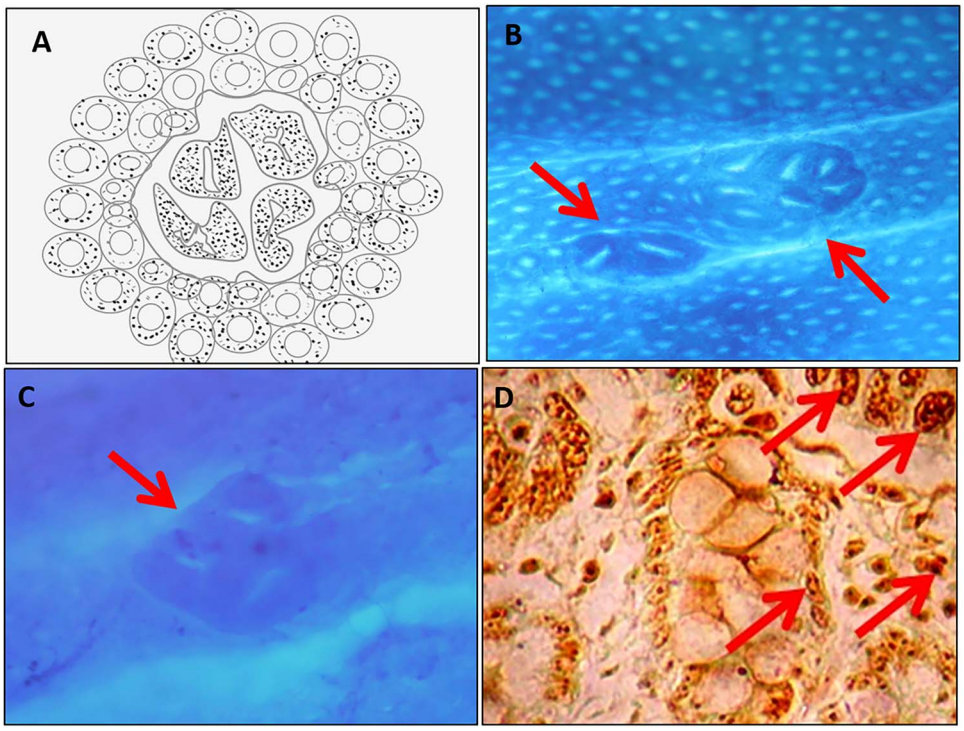

Figure 4: Preneoplastic lesions. (**A**) Schematic diagram of aberrant crypt foci. (**B**) Methylene blue-stained aberrant crypt foci. (**C**) Dysplastic aberrant crypt foci. (**D**) Argyrophilic nucleolar organizing regions.

increase the cleavage of nontoxic to toxic compound, associated with harmful effects on the host, which in particular may lead to the initiation and/or promotion of carcinogenesis [86].

In the same context, β-glucosidase and β-galactosidase are responsible for the generation of aglycone from plant glycosides; therefore these enzymes may hydrolyze DMH to get its toxic metabolite MAM [87].

Mucinase is another one important enzyme hydrolysis protective mucin in the colon. Mucins, basically proteoglycan, form gel, coating the intestinal mucosa and functioning as chemical and mechanical barrier against bacteria, viruses, and toxins [88]. Mucinase activity is altered by the carcinogen exposure in experimental studies; in turn amplified mucinase activities cleave large area of mucin in the colon. Therefore, direct exposure of carcinogen to colonic cells turns the normal cells to malignant one [89].

Nitroreductase (generally distributed in bacteria) is involved in the conversion of dinitrotoluene, nitrobenzenes, and nitropyrenes (aromatic compounds) to amines, which often exhibit toxic, mutagenic, or carcinogenic activities [90].

Fecal sulfatase activity should also be considered in the desulfation of conjugated toxins and in the degradation of sulfated mucins. Changes in the expression of sulfated molecules such as mucins and other glycoconjugates have been demonstrated in the transformed colonic epithelial cells.

The overall types of bacteria in the intestinal tract may be important in evaluating the relationship between diet and intestinal flora rather than the actual number of bacterial organisms [91]. The metabolic activity of intestinal microflora can be modified by dietary factors, for example, they can specifically play a significant role in the conversion of bile acids and neutral sterols to form relative metabolites that can act as promoters [86]. Therefore, fecal bacterial enzymes are known to be involved in the conversion of procarcinogen to proximal carcinogens.

Alterations in Glycoconjugates Levels

Alteration of glycoconjugates in cancerous tissue may be quantitative, qualitative, or both. Glycoproteins of the plasma membrane play an important role in cell-to-cell contact, growth regulation, and binding sites for hormones and lectins.

In tumor cells, alteration of glycoconjugates like hexoses, hexosamine, and fucose may also contribute to the aberrant cell-cell recognition, cell adhesion, antigenicity, and invasiveness of malignant cells [92]. Carbohydrates are early considered as energy sources and structural sources, and proteolytic agents have been slowly refocused on the fact that they have diagnostic and therapeutic potential. New scientific evidence has suggested a relationship between carbohydrate structure and many biological functions.

Oncogenes are known to induce the expression of Golgi β-1,6 N-acetylglucosaminyl transferase in many cell types, leading to increased cellular motility and decreased substratum adhesion [93]. Aranganathan *et al.* [94] reported that the level of protein-bound hexoses and fucose is elevated in cancerous conditions.

The sialic acid and hexosamine were remarkably lowered in the colon and elevated in the liver of carcinogen-exposed rats [95]. Decreased activity of some glycosyltransferase leads to the reduced level of carbohydrate content in tumor tissues [96].

Role of Xenobiotic-Metabolizing Enzymes

It is generally accepted that the biotransformation of substances foreign to the body (xenobiotics) including drugs is divided into phases I and II.

Cytochrome P450 (CYP), a phase I drug-metabolizing enzyme, is playing a major role in the activation of carcinogens [97]. Since metabolic activation is required for many carcinogens before binding to DNA, individuals with an elevated metabolic capacity to activate specific carcinogens may be at an increased risk of cancer [98]. Therefore, CYP-dependent metabolism not only involving in exert toxicity ot carcinogenicity, but also the targets for phase II enzyme dependent conjugation reactions are formed, rendering them inactive polar products suitable for excretion via the kidneys.

The main role of phase II enzymes is to perform conjugating reactions, such as glucuronidation, sulfation, methylation, acetylation, glutathione, and amino acid conjugation; the respective conjugates are more hydrophilic than the parent compounds [99, 100]. Metabolism of DMH involves several xenobiotic-metabolizing enzymes, which activates the procarcinogen. The rat liver cytochrome P450 2E1 (CYP2E1) metabolizes the DMH metabolites azoxymethane (AOM) and methylazoxymethanol (MAM) in an *in vitro* microsomal system [101]. Elevated levels of CYP2E1 were reported in DMH-induced colon cancer [102]. In addition, rats administered with β-naphthoflavone, a potent inducer of CYP1A1, exhibited enhanced formation of the promutagenic DNA adduct O6-methylguanine after DMH treatment [103]. It is evident that procarcinogen DMH induces CYP2E1; therefore conversion occurs and the formation of ultimate carcinogen through the biotransformation.

GSH reacts spontaneously or via catalysis by glutathione-S-transferases (GSTs) with numerous activated carcinogens [104], rendering them excretable and less toxic. Decrease in the activities of GST and DTD in the carcinogen-exposed rats shows (i) the conjugation process utilizes excess amount of these enzymes and (ii) the exposure to carcinogen and other tumor promoters reduces the protective ability of these enzymes against cell damage by other metabolites [59].

An increase in phase II detoxification enzymes might be considered to be beneficial, because this could enhance the excretion of carcinogens. Secondary metabolites from plant kingdom are known to block the cytochrome P450 system responsible for converting carcinogenic agents into forms capable of covalent binding with DNA [105].

Modulation of Molecular Pathways

The APC gene encrypts a multifactorial protein that may participate in many cellular events such as cell adhesion and migration, signal transduction, microtubule assembly, and chromosome segregation. However, even though all of these functions are possibly linked with cancer, it seems that the major tumor-suppressing function of APC resides in its ability to precisely regulate intracellular β-catenin levels [106–108].

Moreover, although the majority of colorectal tumors carry mutations in APC, those with an integral APC gene were found to contain activating mutations in β-catenin that alter functionally significant phosphorylation sites [107, 109]. In addition, mutations in other members of the Wnt pathway have been shown to be associated with cancer including conduction [110] and axin [111, 112]. Presently, p53 is known to play a key role in practically all types of human cancers, and the mutation or loss of the p53 gene can be identified in more than 50% of all human cancer cases worldwide.

The p53 mutational spectra also can indicate that a particular cancer did not result from an environmental carcinogen but instead was caused by endogenous mutagenesis. The high frequency of C to T transitions at CpG dinucleotides in colon carcinomas [113] is consistent with mutagenesis by endogenous deamination mechanisms. A C to T transition would be generated by spontaneous deamination of 5-methylcytosine [114] or by enzymatic deamination of cytosine by DNA (cytosine-5)-methyl transferase when S-adenosylmethionine is in limiting concentration (or by both mechanisms) [115]. Because oxygen radicals enhance the rate of deamination of deoxynucleotides [116, 117], chronic inflammation and nitric oxide generated by nitric oxide synthases may explain why rats that inhale particulate materials, which cause inflammation but do not act directly on DNA, have a high incidence of lung cancer [118].

In vivo studies have also shown that p53 mutation has a role in colon cancer progression, but this becomes important only in the late stages of the disease [119]. Thus p53 mutation observed in the advanced tumor stage of colon cancer indicates that p53 mutation may be a late event contributing to tumor progression [120]. The ability of p53 to serve as a prognostic marker has been extensively studied in colorectal cancer, with most studies focusing on increased immunohistochemical staining [121].

Exposure to ultraviolet light is a common carcinogen and is correlated with transition mutations at dipyrimidine sites [122]; dietary aflatoxin B1 exposure is associated with G:C to T:A transversions that bring to the serine substitution at residue 249 of p53 in hepatocellular carcinoma [123, 124]; and exposure to cigarette smoke is correlated with G:C to T:A transversions in lung carcinomas [125].

Inflammation, proliferation, and apoptotic markers (COX-2)

Epidemiologic studies have proved that individuals who consume nonsteroidal anti-inflammatory drugs on a regular basis compared with those not taking these agents have 40–50% reduction in mortality from colon cancer. All of these drugs have one unique property that is their ability to inhibit COX, a key enzyme involved in the conversion of arachidonic acid to prostaglandins. Raised levels of prostaglandin E-2 (PGE-2), the predominant prostaglandin produced by COX-2, are detected in colon cancer tissues and in macrophages derived from colon cancer, whereas PGE-2 is only moderately present in normal mucosa [126]. Elevated COX-2 expression has been found in colon cancer tissues from subjects with clinically diagnosed colorectal cancer [127–129]. There are two mechanisms involved in colon cancer angiogenesis: (i) COX-2 can modulate the production of angiogenic factors by colon cancer cells and (ii) COX-1 regulates angiogenesis in endothelial cells [130].

The COX-2 gene may be regulated by hypoxia via the activation of Nuclear factor κ-B (NF-κB) in human vascular endothelial cells [131], while COX-2 overexpression in cancerous epithelial cells may be induced through the target of normal APC-the β-catenin oncoprotein [132]. The principle role of wild-type APC involves the binding and degradation of β-catenin. Most colorectal cancers have loss of function mutations in the adenomatous polyposis coli (APC) tumor suppressor gene. This leads to the accumulation of β-catenin. COX-2 can be downregulated by wild-type APC induction and upregulated by nuclear accumulation of β-catenin in the presence of mutant APC. The most common mutation in colon cancer is APC gene mutation. Thus, it would suggest a direct role of APC loss in COX-2 overexpression.

Carcinogen exposure makes the upregulation of COX-2 protein expression and also the COX-2 mRNA in rat colon tissues. It may be due to the loss of function of APC and accumulation of

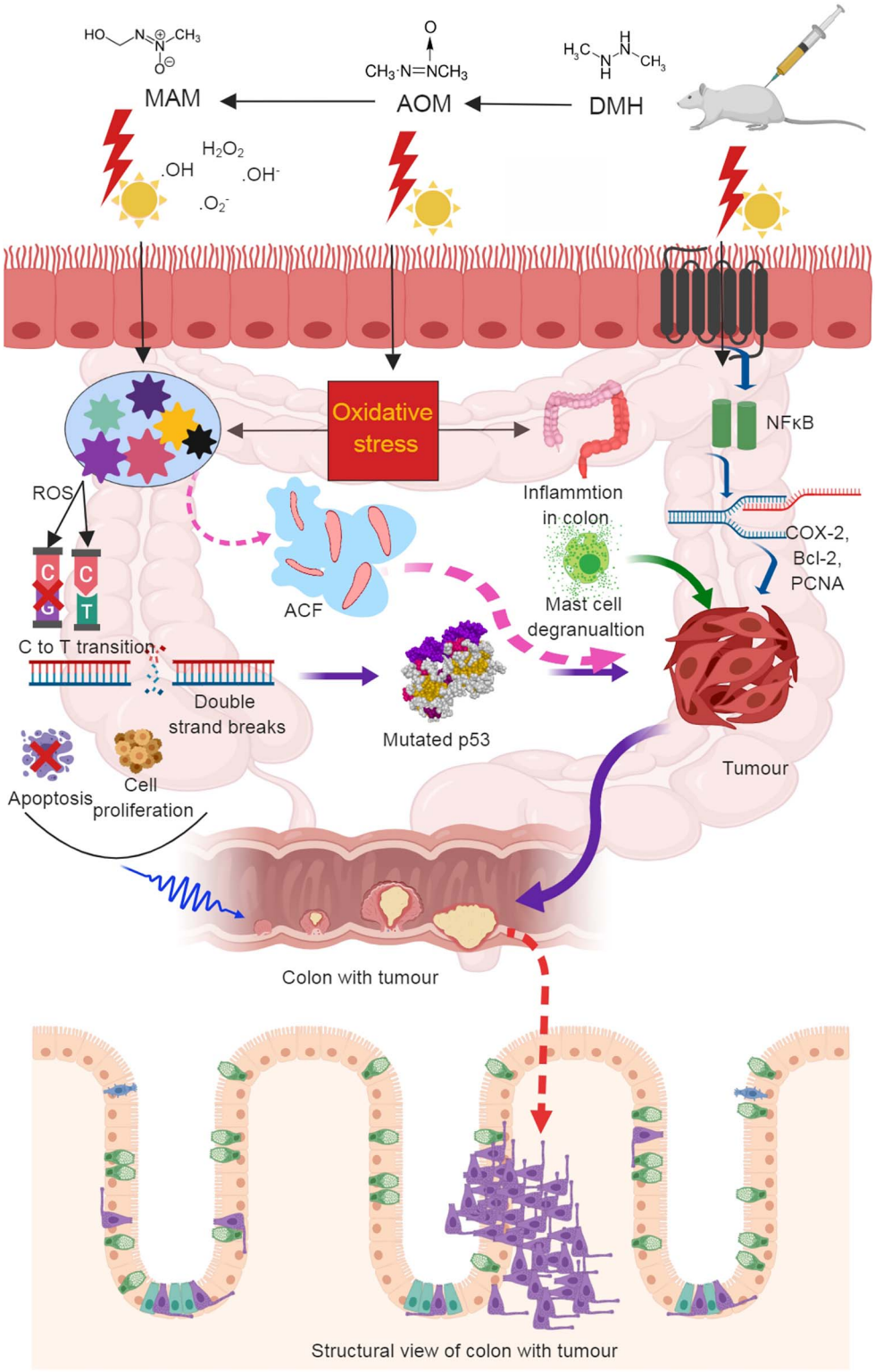

Figure 5: Schematic expression of overall mechanism of DMH-induced carcinogenesis.

β-catenin in carcinogen exposure. COX-2 expression in human tumors can be induced by various growth factors, cytokines, oncogenes, and other factors. IL-1b has been reported to upregulate COX-2 expression in human colorectal cancer cells via multiple signaling pathways [133].

Overexpression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli. Overexpression of Bcl-2 prevented the efflux of cytochrome c from the mitochondria and the initiation of apoptosis. Thus, one possible role of Bcl-2 in prevention of apoptosis is to block cytochrome c release

from mitochondria. Moreover, dysregulation of cell death genes leading to overexpression of Bcl-2 or reduction in Bax expression, for example, would alter the Bcl-2/Bax ratio which is considered to be anticarcinogenic and vice versa [134].

Nuclear factor κ-B

Activation of NF-κB must be a tightly regulated event. During normal conditions, NF-κB become activate after an appropriate stimuli and upregulated in the transcription of target genes. Then NF-κB came back to inactive state. In cancerous conditions, different types of molecular alterations trigger impaired regulation of NF-κB. In this context, NF-κB constitutively activated leads to deregulated expression of genes under NF-κB control. Alternations in all these processes participate in the development and progression of cancer [135]. Deregulated NF-κB expression has been found in a number of different types of cancer. NF-κB regulates genes responsible for all survival, proliferation, inhibiting apoptosis, and mediate invasion and metastasis. Loss of function of APC genes results in the activation of β-catenin signaling, the foremost step in the development of colon cancer, which was observed in APC Min$^+$ mice model.

It has been reported that TNF receptor superfamily member 19 (TNFRSF19) is a β-catenin target gene, and TNFRSF19 receptor molecule-associated activation of NF-κB signaling has demonstrated that β-catenin may regulate NF-κB activity via TNFRSF19; activation of NF-κB activity has also been observed in the APCMin$^+$ mice model, which was inhibited by Riccardin D [136]. Novel anticancer drugs induce apoptosis in cancer cells, and apoptotic dysfunction leads to the progression of cancer [137]. Usually, anticancer drug treatment results in the activation of caspases, which effectively implement apoptosis. Polyphenols can act as antioxidants as well as prooxidants depending on the tumor environment. Oxidation of polyphenols produces O_2, H_2O_2, and a complex mixture of semiquinones and quinones, all of which are potentially cytotoxic [138–141].

Since NF-κB activation is the result of a multi-step signaling pathway, these compounds may target different points of the signaling process. For example, some anti-inflammatory drugs may inhibit NF-κB by interfering with IKK activity [142]. Other substances such as curcumin, trans-resveratrol, or parthenolide are natural compounds that have been demonstrated to inhibit IKK activity [143]. NF-κB inhibition is also considered to be an important therapeutic target in CRC. Thus, it may be hypothesized that the inhibition of NF-κB by Riccardin D maybe a pivotal mechanism of its effects in chemotherapy for CRC with the APC mutation.

Conclusion and Future Perspectives

Rodent model for colon cancer is one of the best ways to understand the underlying mechanism of colon carcinogenesis and its progression, as well as the comprehensive treatment approaches.

DMH-induced colon carcinogenesis is influenced by age, sex, strain, bodyweight, and, most predominantly, by the diet. From the different routes of DMH administration, we conclude that a single dose of s.c. injection to rats developed a preneoplastic lesions, whereas other routes of administration do not produce feasible results. The same pattern of tumor induction is observed in mice; however the hamsters are slightly resistant to DMH carcinogenicity even administered as s.c. Various studies revealed that high-fat diet plays a major role in the progression of colon tumor in rodents. Rats and mice are the sensitive species for DMH carcinogenicity; however, the latency period is crucial. There is solid proof that the colon tumor induced by DMH showed a very high tumor growth and malignancy behavior by its histopathological evidence and metastatic nature Fig. 5.

Evidenced from many animal studies, it is very clear that natural products from plant kingdom have the strong chemopreventive activities against DMH-induced colon carcinogenesis. These compounds may possess at least one of the following (Table 1) properties such as blocking the initiation of tumor in the local site, inhibiting the progression by influencing in the tumor metabolism, preventing the binding with nucleic acids, and stimulating DNA repair mechanism and inhibiting the cell proliferation and inducing apoptosis. In future, the mechanism behind the tumor microenvironment and pro- and antioxidant roles of natural products need to be explored.

Conflict of Interests

The authors declare that they have no competing interests.

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Authors' Contributions

K.V., R.V. and A.M. prepared the first draft of this manuscript, and N.M.I. and R.P. are involved in critical reading, correcting, and idea for schematic figures.

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