RESEARCH ARTICLE

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Potential Prophylactic Effect of Berberine against Rat Colon Carcinoma Induce by 1,2-Dimethyl Hydrazine

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Abstract

Introduction: Colon Cancer remains one of the major worldwide causes of cancer related morbidity and mortality in both genders. Berberine (BBR), a major component of alkaloids that possess a variety of pharmacological properties. **Objective:** This study shows the ameliorating roles of berberine on 1,2 Di methyl hydrazine (DMH) induced colon cancer in male Swiss albino rats. **Methods:** The rats were segregated into four groups: group 1, control rats; group 2, rats were orally received berberine (75 mg/kg b.wt./day) daily for ten weeks; group 3,rats were subcutaneously injected with DMH (20 mg/kg b.wt) once a week for 8 weeks ,group 4, rats were treated firstly with berberine for two weeks before DMH intoxication and concurrently with DMH over 8 weeks. **Result:** DMH injection decreased the antioxidants levels (GSH and SOD) and increased inflammatory markers (MPO, MAPK and COX-2). Moreover, it downregulated apoptotic markers (Caspase-3 and P53) expression that confirmed by colon cell proliferation. The prophylactic effect of berberine was noticed as its pre-and co-administration increased antioxidants status and apoptotic markers expression that associated with inflammatory markers down-regulation with absence of proliferated colon cells. **Conclusion:** Therefore, the overall findings proved that the anti-proliferative effect of berberine return to its antioxidants and anti-inflammatory properties that activated the programmed cell death process.

Keywords: Apoptotic markers- antioxidant- MAPK- myeloperoxidase- cyclooxygenase-2

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Introduction

Colon Cancer (CC) is one of the leading cause of cancer related mortality and morbidity in human in developed countries (Jamal et al., 2011). The pathogenesis of CC is very complex and diverse and also influenced by multiple factors some of them related to diet and life style while others related to genetic factors (Marchand et al., 1997).

most of genetic CC follow the chromosomal instability (CIN) pathway that characterized by loss of heterozygosis (LOH) and gross of chromosome abnormality. Other systems and pathways are involved in the pathogenic of CC including microsatellite instability (MSI), abnormal DNA methylation, inflammation and micro RNA can actively contribute to the CC (Lin et al.,2003). CC also causes by chemical agent such as 1,2-Dimethyl hydrazine (DMH) which consider as one of the most common chemical that widely used to induce colon carcinoma in rats. DMH metabolically activated in liver by series of reaction through intermediate azoxymethane (AOM) and methylazoxymethanol (MAM) to the ultimate carcinogenic metabolite, highly reactive methyl-di-azonym ion. MAM can be excreted into the bile and transported to colon or enter directly into epithelial cells of colon from blood circulation. (Fiala and Stathopoulos, 1984). This ultimate carcinogenic metabolite of DMH is responsible for methylation of the DNA bases in colon epithelium cells result in escaping from apoptosis that lead to proliferation and form aberrant crypt foci (ACF) that lead to adenoma then forming carcinoma (Magnuson et al., 2000).

Berberine (BBR) is a natural isoquinoline alkaloid widely uses in traditional medicine. BBR possess aviary of pharmacological properties against diarrhea, hyperlipidemia, obesity, diabetic and hypertension. Several in vitro studies proved that BBR has anti-inflammatory and antioxidants properties beside that it has anti-proliferative effect toward cancer cell lines through direct interact with nucleic acid and several proteins (Zhang et al., 2010).

Therefore, this study was designated to investigate the prophylactic effect of berberine against DMH induced colon cancer through tracking oxidative stress, inflammatory and apoptotic markers.

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Materials and Methods

Animals and experimental design

The present study was carried out using 40 adult male Swiss albino rats (120 ±20 g) obtained from Nile Company for Pharmaceuticals and Chemical Industries (Cairo, Egypt). The animals were kept in standard plastic cages in a well-ventilated room. Rats were naïve to DMH and were maintained with free access to laboratory pellet chow with water ad libitum under controlled conditions of temperature (27 ± 2°C) and humidity (60 ± 5%) with 12 h light/ 12 h dark cycles. The experimental protocol was carried out according to the Guide for the Care and Use of Laboratory Animals (NIH, 1985).

Rats were equally divided into four groups; group1; control group; rats were received daily one ml of physiological saline by gastric gavage; Group II, BBR control group, rats were daily received 75 mg berberine /Kg BW by gastric gavage for continuous 10 weeks.; Group III, DMH group, rats were subcutaneously injected with 20 mg DMH/kg BW once a week for 8 consecutive weeks; Group IV, BBR+DMH group, rats received 75 mg berberine /Kg BW by gastric gavage for 15 days prior DMH intoxication and concurrently with DMH over eight weeks.

At the end of experimental period, the animals were fasted overnight, anesthetized with diethyl ether then sacrificed by cervical decapitation. Colon tissue was existed immediately and rinsed in ice cold physiological saline, blotted dry on filter paper and weighted.

Colon tissue was homogenized in 0.1 M Tris –HCl buffer (pH 7.4) using tissue homogenizer with Teflon pestle at 4 ^{0}C, then centrifuged at 3000 rpm for ten minutes. A portion of colon tissue was fixed in 10% formalin saline for 24 hours for histological examination.

Biochemical and Molecular investigations

Oxidative stress parameters

Malondialdehyde (MDA) level was assessed in freshly drawn samples by means of the thiobarbituric acid (TBA) reaction, according to (Yoshioka et al., 1979) in which the formed pink colored complex was measured at 532 nm. The concentration of MDA was calculated in nmol/g wet tissue using this formula: A/0.156*dilution where 0.156 is MDA extinction coefficient.

Glutathione (GSH) level and SOD activity were measured by using standardized methods (Beutler et al., 1963; Minami and Yoshikawa, 1979) respectively.

Inflammatory markers

Myeloperoxidase (MPO)was assessed according to (Kettle and Winterbourn, 1994) by using commercial kit. Cyclooxygenase 2 (COX-2) activity was determined by using rat ELISA Kit (My Biosource Inc., San Diego, California, USA) according to manufacture instructions.

P38 mitogen-activated protein kinases (p38MAPK) was measured using western blot technique. In which, 100 mg colon tissue was mixed with one ml Trizol to extract protein. The protein concentration was measured by the Bradford method (Bradford, 1976). Twenty micrograms of protein per lane were separated with 10% SDS polyacrylamide gel electrophoresis gels and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were then incubated at room temperature for 2 h with blocking solution comprised of 5% nonfat dried milk in 10mMTris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween20.Membranes were incubated overnight at 4°C with the indicated primary antibodies (P38 MAPK and beta-actin) and then incubated with a mouse anti-rabbit secondary monoclonal antibody conjugated to horseradish peroxidase at room temperature for 2 h. After each incubation, the membranes were washed four times with 10mM Tris-HCl, pH 7.5, 100mM NaCl, and 0.1% Tween 20 at room temperature Chemiluminescence detection was performed with the Amersham detection kit according to the manufacturer's protocols and exposed to X-ray film. The amount of studied protein was quantified by densitometric analysis of the autoradiograms using a scanning laser densitometer (Biomed Instrument Inc., USA). Results were expressed as arbitrary units after normalization for β-actin protein expression.

Apoptotic markers

Active caspase-3 level in colon tissue homogenates was colorimetrically detected by using commercial kit according to manufacture instructions.

P53 gene expression was measured by using qPCR. Briefly, about 30mg of tissue was used to extract RNA by using SV total RNA extraction kit (Promega, USA). The obtained RNA concentration was determined spectrophotometrically at 260 nm. The extracted RNA was reverse transcribed into cDNA by using Random hexamers. The P53 and B-actin quantitative expression were conducted by using Sybr green and specific primers. The primers sequences were; P53(F:5-CCTCTTGCTTCTCTTT TCCTATCC-3 R:5-CTTGGTCTCCTCCACCGCTTCTTG-3)and β-Actin(F: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'). Each primer (forward and reverse) concentration in the mixture was 5 pmol/ µl. The qPCR condition was 40 cycles of denaturation at 95 ^{o}C for 15 sec; Annealing at 60 ^{o}C for 60 sec and Extension at 72 ^{o}C for 60 sec. Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the β-Actin gene as an invariant endogenous control (reference gene). The relative quantification was then calculated by the expression $2^{-\Delta\Delta Ct}$.

Histopathological examination

The fixed colon tissue was washed in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) that were used for dehydration step. Specimens were cleared in xylene and embedded in paraffin at 56 ^{o}C in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness using microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin stain for routine examination through the light electric microscope (Banchroft et al., 1996).

Statistical analysis

All the grouped data were significantly evaluated with SPSS/10 software. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test p values of less than 0.05 were considered to indicate statistical significance. All the results were expressed as Mean +/- SD for ten rats in each group.

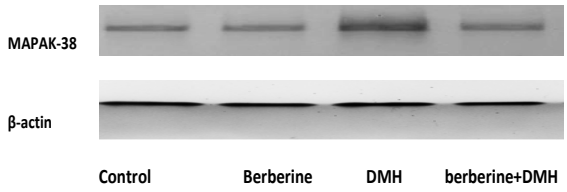

Figure 1. Effect of Berberine and/ or DMH on Western Blot of MAPAK-38 and B-actin

Results

The administration of berberine to healthy rats slightly decreased MDA level than this of control one while increased both GSH level and SOD activity to the maximum limits, at $p<0.05$. On one hand, DMH injection for eight weeks significantly decreased MDH and GSH levels as well as SOD activity, these values were the lowest among all groups, $p<0.05$. The co-administration of berberine with DMH significantly increased MDA level than that of DMH group level which still lower than both control and berberine groups' levels while it successfully normalized of GSH level and SOD activity, at $p<0.05$ (Table 1).

Table (2) indicates that berberine group had the same inflammatory markers (MPO and COX activities as well asP38 MAPK level (figure1)as control one, at $p<0.05$. DMH group showed the highest activities of inflammatory markers, at $p<0.05$. The co-administration of berberine with DMH improved the inflammatory markers but did not normalize them, at $p<0.05$.

Data represented in Table (3) indicate that berberine administration for healthy animal had no effect on apoptotic markers (P53 and caspase 3), at $p<0.05$, when comparing with control levels. DMH injection significantly decreased both P53 and caspase 3 to the lowest levels among all tested groups, at $p<0.05$. The co-administration of berberine with DMH improved the both P53 and caspase 3 but did not normalize them, at $p<0.05$.

Figure (2C and D) show that berberine administration did not alter the normal histological structure of the

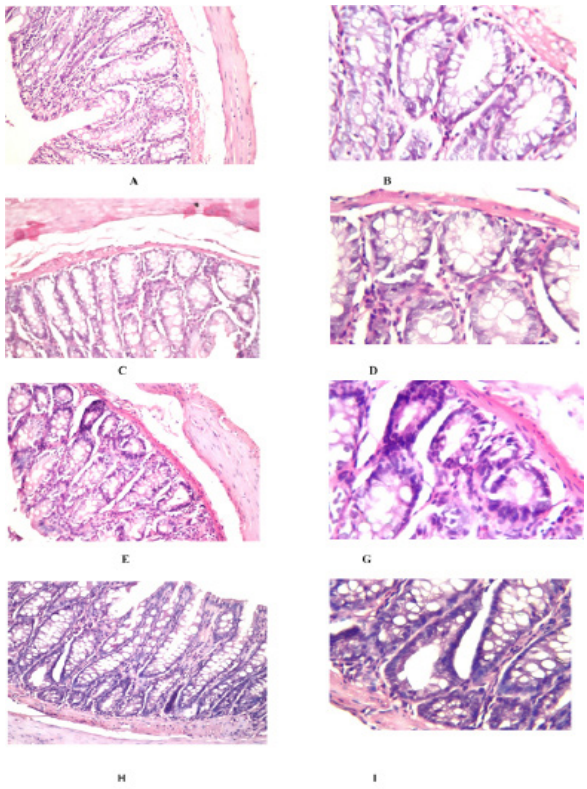

Figure 2. Effect of Berberine and/ or DMH on Colon Histology. Hematoxylin-eosin staining of liver at magnification 40x (A, C, E, H) and 80x (B, D, G, I) of light microscope for (A&B) control group, (C&D) Berberine group, (E&G) DMH group, (H&I) Berberine +DMH group.

Table 1. Effect of Berberine and/ or DMH on Oxidative Stress Parameters in Colon Tissue of the Rats

Groups	MAD (nmole/g tissue)	GSH (mg /g tissue)	SOD (U/ mg tissue)
Control	11.7±0.33#	0.75±0.03#	1.34±0.04#
Berberine	10.80±0.10*#	0.81±0.01*#	1.69±0.05*#
DMH	5.63±0.05*	0.70±0.01*	0.84±0.03*
Berberine + DMH	6.61±0.06*#	0.78±0.05#	1.25±0.09#

Values are expressed as means ± SD; n = 10 for each group, Means with * are significantly different with control mean, at $p < 0.05$, Means with # are significantly different with DMH mean, at $p < 0.05$

Table 2. Effect of Berberine and/ or DMH on Inflammatory Biomarkers in Colon Tissue of the Rats

Groups	MPO activity (U/mg tissue)	COX-2 Activity (ng / mg tissue)	P38-MAPK (Relative protein expression)
Control	2.1±0.01#	1.04±0.04#	1.02±0.02#
Berberine	2.2±0.30#	1.01±0.01#	1.03±0.02#
DMH	13.4±2.90*	15.4±0.75*	11.32±0.8*
Berberine + DMH	5.4±1.10*#	6.55±0.25*#	4.95±0.25*#

Values are expressed as means ± SD; n = 10 for each group, Means with * are significantly different with control mean, at $p < 0.05$, Means with # are significantly different with DMH mean, at $p < 0.05$

Table 3. Effect of Berberine and/ or DMH on Apoptotic Biomarker in Colon Tissue of the Rats

Groups	P53 (Relative gene expression)	Caspase-3 activity (U/g tissue)
Control	1.01±0.01#	2.55±0.15#
Berberine	1.04±0.04#	2.43±0.53#
DMH	0.24±0.03*	0.59 ±0.10*
Berberine + DMH	0.83±0.07*#	1.50±0.14*#

Values are expressed as means ± SD; n = 10 for each group, Means with * are significantly different with control mean, at $p < 0.05$, Means with # are significantly different with DMH mean, at $p < 0.05$

mucosa and glandular structure when compared to control one (Figure 2A and B). DMH administration leaded to loss of goblet cells formation in mucosal layer which associated with dysplasia in the lining glandular structure. Moreover, hyperchromachia in the nuclei of the lining epithelium as well as cellular pleomorphism were recorded (Figure 2E and F). Finally, the co-administration of berberine with DMH prevented the DMH adverse effects on colon histology as figure 1g and H showed normal colon architecture (Figure 2G and H).

Discussion

Despite the anticancer effect of berberine in vitro and in vivo, the mechanism by which it exerts the antineoplastic actionis not fully understood. Therefore, in this study, the antioxidants, anti-inflammatory and apoptotic effects of berberine toward DMH -induced colon carcinoma in rats were investigated.

Oxidative stress plays an important role in all stages of chemical carcinogenesis and tumorigenesis (Mandal, 2017). Hepatocytes metabolized DMH to a methyl-free radical which participated in lipid peroxidation (LPO)initiation (Bobek et al., 2000). LPO altered membrane permeability, oxidized membrane protein, damaged cellular macromolecules and finally triggered cell death (Sharma and Dubey, 2007).

MDA, LPO end product, was found to be increased in colon cancer tissues (Skrzydewska et al ., 2001) but, the present study results showed a marked reduction in colon cancer MDA level. This finding could be attributed to the elevation of cell proliferation, cellular resistance and/or susceptibility of target organs to free radical attack reduction.

One of cancer self-defense is the reduction of antioxidants system and this is accounted as characteristic findings in malignant transformation (Rovereet al., 2000; Ray et al., 2000). In agreement with this theory, the present results showed a depletion in detoxifying antioxidants (GSH, SOD). GSH exhibits anticarcinogenic and antioxidant properties (Lii et al,1998; Jagetia and Rao, 2017). Also, this colon cancer tissue GSH depletion may be due to its highly utilization to counteract LPO (Devasena et al., 2003). Furthermore, SOD which is responsible for the elimination of the superoxide (O_2^-) radical, was found low in cancer tissue with high proliferation index (Satomi et al., 1995; Jagetia and Rao, 2017).

On the other hand, the supplementation of berberine resulted in increased levels of colon tissue MDA and GSH levels and SOD activity compared to the control group. These elevations may be due to the free radical scavenging property of berberine and consequently decreased utilization of the antioxidant enzymes. The anticancer effect of berberine returned to its ability to act as potent free radical scavenger and antioxidant (Dkhil, 2014).

Jagetia and Rao (2017) reported that berberine chloride increased MDA level in Ehrlich ascites carcinoma cells (EAC) which permits the tumor cell killing by changing the membrane fluidity and increasing DNA damaging.

Inflammation is related to oxidative stress, where myeloperoxidase (MPO)which is present in neutrophils, is responsible for H_2O_2 production. Colon cancer and chronic intestinal inflammation is linked to each other, where inflammation influences tumor initiation and promotion (Takahashi et al., 2015). The present study found that colon cancer group showed an elevation in the intestinal inflammatory markers (MPO and COX-2) through P38-MAPK-mediating response. Therefore, the level of MPO activity is directly proportional to the neutrophil concentration in the inflamed tissue. Therefore, a measurement of MPO activity has been considered a quantitative and sensitive assay for acute intestinal inflammation. In addition, increased MPO activity has been reported to be an index of neutrophil infiltration and inflammation (Choudhary et al., 2001).

COX-2, an important inflammatory marker, plays a central role in colon toxicity and polyp formation as itwas found to be over-expressed in colon cancer, In the current investigation, DMH treated group showed the highest COX2 level. COX-2 is mainly expressed in cancerous condition via the activation of inflammatory cytokines. It directs cancer cell proliferation by inhibiting apoptosis and enhancing cancer-induced angiogenesis (Wendum et al., 2004).

There are several upstream kinase pathways responsible for transcriptional regulation of COX-2 including mitogen activated protein kinases (MAPKs). MAPKs are composed of extracellular receptor kinase (ERK), p38 kinase and c-jun NH2 terminal kinase (JNK) (Dhillon et al., 2007). The current data proved that DMH injection increased the protein level of active phosphorylated p38 MAPK. The p38 mitogen-activated protein kinase pathway not only regulates the production of inflammatory mediators, but also controls processes related to tissue homeostasis, such as cell proliferation, differentiation and survival, which are often disrupted during malignant transformation (Gupta and Nebreda, 2015). The p38 mitogen-activated protein kinase pathway not only regulates the production of inflammatory mediators, but also controls processes related to tissue homeostasis, such as cell proliferation, differentiation and survival, which are often disrupted during malignant transformation.

The anti-inflammatory properties of berberine has been shown in this study where its administration prevented DMH adverse effect in inflammatory pathways as it improved the levels of COX-2 and P38 MAPK as well as MPO activity. Anti inflammatory activity of berberine has been demonstrated in experimental colitis in vitro and in vivo (Kawano et al., 2015). Moreover, major anti-carcinogenic effects of certain drugs occur through the inhibition of COX- 2 and selective inhibitors of COX2 reduce prostaglandins synthesis, thereby attenuating the inflammation and progression of cancer (Watanabe et al., 2000). The reduction that occurred in COX-2 level may be due to the induction of the cell cycle regulating protein p21, which is implicated as a possible mechanism of chemoprevention of colorectal carcinogenesis (Huls et al., 2003). Impressively, it was shown that berberine possesses anti-inflammatory activity and suppresses proinflammatory responses by inhibiting mitogen-activated protein kinase signaling (Jabbarzadeh et al., 2014).

The elevation of COX-2 level reflected the elevation of prostaglandins production which confirmed by P38-MAPK level that precedes the upregulation of Bcl-2, anti-apoptotic protein or inhibited P53, apoptotic marker (Sobolewski et al., 2010).

P53 role as a cell cycle regulator is through stop the cell cycle in G1 phase which caused by the increase transcription of p21 that would inhibit the formation of cyclin-CDK complexes (Xu et al., 2010).

Interestingly, the present results indicate that DMH injection down-regulated p53 expression. This finding agrees with study of Kilari et al., (2016) who state that the DMH is a mutagenic compound that causes P53 mutations in colonic epithelial cells. Induction of apoptosis is considered as one of the possible mechanisms of inhibition of cancer development, and many chemopreventive agents have been shown to act through the induction of apoptosis in their inhibition of the carcinogenic process. Several investigations indicate that apoptosis may represent a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells that have improperly been induced to proliferate by factors such as carcinogens (Katiyar et al., 2012). Our present set of data suggest that the induction of apoptosis in colon of rats injected with DMH may be one of the mechanisms by which berberine exerts its anti-cancer effects. The current study found a significant upregulation in the expression of P53 gene in colon of rats injected with DMH concurrent with berberine compared with DMH- injected group.

The cell death receptor pathway is mediated distinctively by active/cleaved caspase-8 that is characterized by binding cell death ligand and cell death receptors followed by activation of caspase-8 and caspase-3 for apoptosis to occur (Liu et al., 2011). The results from this study indicated a decrease in caspase-3 in DMH treated rats. The obtained results concerning the caspase-3 is same line with the results of (Tarek et al., 2014), who attributed the decrease in caspase-3 activity to the overexpression of caspase-3 inhibitors and surviving in tumor cells (Krajewski et al., 1995).

Administration of berberine to DMH-injected rats increased caspase-3 activity. The same results were obtained by Ho et al., (2009), who reported that berberine administration enhanced the cleaved of caspase-8, -9 and -3 in human tongue squamous carcinoma cells (SCC-4 cells). Moreover, the study of Ho et al., (2009) indicated that the increase in caspase-9 cleaved led to subsequent activation of the downstream caspase-3 (an apoptotic executioner). Also, their study reported that berberine promoted the expression of Apaf-1 which led to promotion of caspase-3 activation thus causing apoptosis. Histological findings revealed that control group showed normal histoarchitecture with mild infiltration of inflammatory cells as well as intact mucosal glandular structure while DMH-treated group exhibited massive infiltration of inflammatory cells in the lamina propria, distorted mucosal glandular architecture along with crypt ablation and crypt abscess formation. Treatment with berberine strongly suppressed the infiltration of inflammatory cells in the mucosal layer, reduced the severity of submucosal edema, crypt abscess formation and crypt ablation induced by DMH in the colon of Wistar rats. Histological findings clearly revealed that berberine has strong anti-inflammatory property. The above-mentioned findings corroborated with the histological data which exhibited the protective effects of berberine against DMH-induced colonic damage.

Based on the biochemical, molecular and histological changes associated with the administration of berberine demonstrated its ability to minimize the frequency of DMH-induced colon carcinoma as through increasing the apoptotic protein and regulating the level of detrimental oxidative stress markers. These findings suggest possible Prophylactic and therapeutic implications in colon carcinogenesis. Cumulatively, the study suggests that administration of berberine has protective and preventable effects towards colon cancer. The present study highly specifies that berberine own anticancer activity and therefore it can be used as effectual therapeutic agent for treatment of colon cancer.

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