Cold exposure and Capsaicin promote 1,2-dimethylhydrazine-induced colon carcinogenesis in rats correlates with extracellular matrix remodeling

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Abstract

Background: Extracellular matrix (ECM) remodeling and stiffening which was shown to correlate with tumor malignancy drives tumor development. Nevertheless, the relationship between ECM remodeling and colorectal cancer (CRC) of progression and aggression imposed by cold and capsaicin exposure remains unclear.

Methods: Male wistar rats were randomly divided into four groups. Except for normal rats, other group rats were given subcutaneously injected with DMH (25mg/kg b.wt.) once a week for 12 weeks. After which, the animals were respectively treated with capsaicin or cold distilled water (0°C) at 10mg/kg b.wt. by gavage for 38 weeks. We investigated the morphology and structure of collagen and elastin using Masson’s trichrome, Picosirius red, and Weigert’s Resorcin-Fuchsin (WFR) stains. Additionally, we evaluated the protein expression level of COLI, COL III, elastin, LOXL2, MMP1, MMP2, MMP9, and TIMP1 by immunohistochemistry and observed the expression of
COL I, COL III, elastin, and LOXL2 in the colon tissues of rats by qPCR.

**Results:** We found that a trend towards the increase of invasive tumors was observed in the cold and capsaicin group. Cold exposure group had a metastasis rate compared with the other groups. Additionally, abnormal accumulation of both collagen and elastin is observed in the cold exposure and capsaicin group. Specifically, collagen quantitative analysis showed increased length, width, angle, and straightness compared with DMH group. Collagen deposition and straightness were significantly increased in the cold exposure group when compared with the capsaicin group. Cold exposure and capsaicin significantly increase the protein levels of COL I, elastin, and LOXL2 along with increases in their mRNA levels in the colon tissues compared with the DMH group, while COL III did not show a significant difference. Furthermore, in immunohistochemical evaluations, there was an increase in MMP1, MMP2, MMP9, TIMP1 staining in cold exposure and capsaicin group as compared to the DMH group.

**Conclusion:** These results suggest that increased stiffness of colonic tissue and the remodeling of ECM mediated by extracellular matrix enzymes resulting from cold and capsaicin exposure predisposing an environment suitable for CRC development and progression. To target ECM in tumor tissue could represent a novel potential therapeutic strategy.

**Keywords:** Colon cancer, cold exposure, capsaicin, extracellular matrix remodeling, extracellular matrix enzymes

1. **Introduction**

Colorectal cancer (CRC), the common cause of cancer deaths in the world, is a multifactorial disease driven by genetic, epigenetic alterations, and environmental factors [1]. Only a minority of colorectal cancer is caused by the accumulation of genetic epigenetic alterations, whereas the majority are linked to environmental factors such as dietary intake, alcohol consumption, and ambient environment[2, 3]. More and more epidemiological data has indicated that cold weather might be associated with an increased occurrence of cancer [4]. Additionally, there is evidence that a positive association between the consumption of chili-pepper and cancer incidence [5, 6]. However, the mechanisms underlying the effects of cold exposure
and capsaicin in DMH-induced CRC tumorigenesis and progression remain poorly understood.

Extracellular matrices (ECM) is a non-cellular structure that is essential for the maintenance of normal tissue and organ function as well as disease pathophysiology [7]. Collagen is one of the major components of the ECM and not only provides cellular components with physical support but also is an important contributor to tumor growth and progression [8]. Tumor progression is accompanied by dysregulation of collagen structure and deposition. Tumor associated-collagen is usually compacted to thick collagen bundles and anisotropic arrangement of relatively straight in the matrix of malignancies compared with healthy tissues [9, 10]. In clinical samples of breast tumors, there is an increase in collagen deposition, a linearization, and thickening of collagen which was linked to poor prognosis and high risk of mortality [11, 12]. Elastin is another important fibrous ECM protein that provides elastic recoil to tissue. Importantly, excessive accumulation of ECM, in particular collagen and elastin, which gradually leads to progressive organ fibrosis [13]. The fibrosis results in tissue stiffness and can predispose tissue to malignancy. Several human studies indicated that patients with liver fibrosis and liver stiffness, positively correlate with risk hepatocellular carcinoma (HCC) [14]. ECM remodeling is mainly orchestrated by ECM modifying enzymes such as lysyl oxidase–like-2 (LOXL2), metalloproteinases (MMP), and tissue-specific matrix metalloproteinase inhibitors (TIMPs) [15]. One of the key factors in ECM remodeling is LOXL2 which is copper-dependent amine oxidase that catalyzes the cross-linking of collagen or elastin in the ECM and thus regulates the tensile strength of tissues. A considerable amount of the literature suggests that dysregulation of LOXL2 causes disorganization and composition of ECM and resulting in many pathological conditions, including fibrosis and cancer [16]. Active LOXL2 is involved in stiffness-associated cancer progression, whereas inhibition of LOXL2 result in less collagen cross-linking and impeded cancer progression [17]. It was also discovered that LOXL2 expression is overexpressed in many types of tumors, and is associated with poor prognosis [18-20]. MMP has implicated in cancer development, progression, invasiveness, and
dissemination by promoting a protumorigenic microenvironment and modulating the ECM and intercellular junctions [21]. Matrix metalloproteinases (MMPs) and endogenous tissue inhibitors of metalloproteinases (TIMPs) are the main enzymes involved in the regulation of ECM remodeling and collagen degradation process [22]. Their expression and activity were upregulated in almost human cancers with disparate changes and this correlates with advanced tumor stage, poor prognosis, and shorten overall survival rate [23, 24]. Subsequent studies have shown that Increased MMP expression/activity or decreased TIMPs could lead to MMP/TIMP imbalance results in various pathological conditions including fibrosis and cancers [25]. Nevertheless, there is relatively little information on ECM remodeling and extracellular matrix enzymes activity in progression of experimental colorectal malignancy.

We have previously shown that cold exposure and long-term administration of capsaicin at a low dose does further promote the development and progression of CRC [26]. However, the specific mechanisms underlying cold and capsaicin exposure affect tumor promotion remained unknown. The purpose of this study was to investigate the effects of cold exposure and capsaicin on the ECM remodeling and extracellular matrix enzymes in DMH-induced CRC. Moreover, we asked whether excessive ECM deposition, in particular collagen and elastin and dysregulation of extracellular matrix enzymes expression and/or secretion in rat treatment with cold exposure could further stiffen the colon tissues and disrupt the intestinal morphogenesis to exacerbate the experimental colorectal malignancy.

2. Material and Method

2.1. Experimental design

Adult male Wistar rats weighing 200-250g (6 weeks old) were obtained from Experimental Animal Center in Guangzhou University of Chinese Medicine. Animals were housed in plastic cages under a controlled environment (24 ± 2 °C, 50 ± 5% humidity, and 12h/12h light-dark cycle) with ad libitum food and water access. All the experimental protocols were approved by the Institutional Animal Ethics Committee of the Guangzhou University of Chinese Medicine (No.20130001). Briefly, after 3 days of acclimation, the animals were randomly assigned into four groups (n=10).
Rats in group A received no treatment and served as control. Five weeks later, rats in groups B-D were administered with subcutaneous injection of DMH (25mg/kg) once a week for 12 weeks. In addition to DMH, Group C rats received cold distilled water (10mg/kg) until the end of 38 weeks. Group D rats were given capsaicin (0.9mg/ml) every day throughout the experiment. By the end of the week, 10 rats from each group were sacrificed.

2.2. Macroscopic evaluation of the incidence of polyps

At the end of the experimental period, rats were sacrificed and colons were incised and washed with physiological saline. Then cleaned colons were cut opened longitudinally and the number of polyps/tumors was carefully counted and later verified with histopathological examination. Microscope was classified as adenomas and adenocarcinomas according to previous criteria described by Hiroshi et al [27]. Tumor incidence is the percentage of rat bearing the indicated type of tumor.

2.3. Histopathological staining

All specimens were fixed in 4% paraformaldehyde solution for 24h and embedded in paraffin and processed by standard histological processing techniques. Serial issue sections (8-μm thick) were obtained from each sample with the microtome and then were stained with hematoxylin and eosin (H&E), picrosirius red, Masson’s trichrome(MT), and Weigert’s Resorcin-Fuschin (WRF). For Picrosirius red staining, sections were stained in picrosirius red solution 0.1) (Sirius red F3B; Sigma-Aldrich Co., St Louis, MO, USA) in a saturated aqueous solution of picric acid for 1 hour at room temperature for collagen bundle staining. Images were subsequently analyzed using ImageJ to calculate the fiber density which was measured as image % area coverage. Masson’s trichrome was performed according to the manufacture’s protocol including Weigert’s Iron Hematoxylin Solution, Ponceau acid fuchsin, and Aniline Blue as reagents. The collagen volume fraction (CVF) was measured by ImageJ software and calculated as the proportion of blue positive areas in the total section areas. The process of Weigert’s Resorcin-Fuschin was use reagents and kits from Solarbio (Beijing, China). Sections were then mounted for observation under polarized light microscopy (NIKON Eclipse Ci, Japan) and light microscopy.
respectively. Three microphotographs of the reticular dermis were taken with a 400× magnification with light microscopy and polarized microscopy, respectively. Digitized images of histological sections obtained under final magnification of × 400 were analyzed using the Image-Pro Plus 4.5 software.

2.4. Collagen Fiber Analysis

CT-FIRE, an open-resource software (http://loci.wisc.edu/software/cifire) was used as previously described to automatically quantify collagen fibers [28]. The quantitative parameters included alignment of collagen fibers as well as individual length, straightness, and width. These features of collagen fibers are widely used to investigate collagen organization in a various of cancer [29]. All the picrosirius red images were converted to 8-bit images and threshold values between 10-255 to eliminate background noise using FIJI ImageJ [30]. These images were uploaded to CT-FIRE, collagen fiber extraction parameters were set to default parameters.

2.5. Real-time quantitative RT-PCR (RT-qPCR)

Total RNA extracts of colon tissues were prepared using TRIZOL reagent (Takara, Japan). RNA (1 µg) was reverse transcribed in a 20 ul reaction mixture using Prime Script RT Master Mix (TaKaRa, Japan). The purity of total RNA was evaluated by measuring the concentration and OD260:280 values with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The mRNA levels of collagen type 1, alpha1 (COL1A1), collagen type 3, alpha1 (COL3A1), LOXL2, and elastin in colon mucosa were assessed using a Step One Plus real-time PCR system (Thermo Fisher Scientific, CFX384TM Real-time System. The relative levels of gene expression were enumerated using the comparative formula 2^ΔΔCt. The primer sequences used in this PCR amplification were as follows:

5'-AGCCATGTACGTCAGCCATCC-3'/3'-ACCCTCATAGATGGGCACAG-5'
for β'-Actin.

5'-AGGCATAAAGGGTCATCGTGCTT-3'/3'-AGTCCATCTTTTGCCAGGAGAACCA-5'for Col1a1.

5'-GGTTGGAGAACATCTATCGATGGTGG-3'/3'-GCTGGAAAGAAGTCTGAGGAAGGG-5'for Col3a1.
5’-AGCCTATAAGCCGGAGCAAC-3’/3’-GTCCCACTTGTACATCGCAGA-5’f
or LOXL2.
5’-CGCCTGTAATGCTCAAATC-3’/3’-AGCAGCTAAAGCAGCGAAGT-5’f
or Elastin.

2.6. Immunohistochemistry

Colonic tissue sections were deparaffinized and rehydrated through a series of xylene and ethanol/water. The sections were placed in a 95°C antigen retrieval solution (Citrate buffer; PH 6.0) for 15min. After cooling retrieval solutions for 20 mins at room temperature, the slides were treated with hydrogen peroxide (H₂O₂) for 10 minutes to block endogenous peroxidase activity. Primary rabbit anti-histone polyclonal antibodies were applied for 14h at 4°C overnight at the following dilutions: COL I (1:500; ab34710; Abcam), COL III (1:200, ab7778; Abcam), LOXL2 (1:400), Elastin (1:600; ab217356; Abcam), MMP1 (1:500; 10371-2-AP; Proteintech), MMP2 (1:200; ab86607; Abcam), MMP9 (1:800; ab38898; Abcam), TIMP1 (1:600,ab61224; Abcam). The next day, Biotin-conjugated secondary antibody and streptavidin-biotin peroxidase were applied each for 20 mins. 3,3’-Diaminobenzidine tetrahydrochloride (0.05%, DAB) was used as the substrate, and nuclear contrast was performed using hematoxylin counterstaining. Each section was analyzed in three different fields using Image Pro Plus software. The density of yellow reflects the expression levels of target proteins. IOD SUM/Area SUM was applied to quantify the relative expression of COL I, COL III, LOXL2, Elastin, MMP1, MMP2, MMP9, TIMP1.

2.7. Statistical analysis

All the data were summarized as mean±standard deviation (SD), and data were analyzed using SPSS 23.0 statistical software. We performed the data with a one-way analysis of variance with the post-hoc comparison by the L.S.D. method and Fisher’s exact test was employed to compare tumor incidence. Differences with values of P<0.05 were considered statistically significant.

Result

3.1 Macroscopic and pathological observation study of colon tumor

No visible colon tumor was found in the normal control. We observed findings
such as colonic mucosal thickening, stiffness and can’t completely tiled on filter paper in the majority of the rats of cold exposure group (Fig. 1C). As shown in Fig.1B, the length of colon in the DMH group and normal group was not significant. However, the length of the colon in the cold exposure and capsaicin group was shorter than that of DMH group. The pathological classification of colonic tumors in each group can be seen in Table 1. There was no difference in the proportion of adenomas among groups. In the DMH-induced group of animals showing most tumors was well-differentiated tubular adenocarcinomas. A trend towards the increase of invasive tumors was observed in the cold and capsaicin group. Histopathological analysis showed that in the cold exposure group, there was an evident malignant transformation in the colon with the features of poor-differentiated mucinous adenocarcinomas, and some of the glands are filled with mucinous material (Fig.2C). In addition, the mesenteric lymph nodes of rats in each group were stained with H&E, and the lymphatic metastasis was observed under the light microscope (Fig.2B). We found that there was no lymphatic metastasis in the DMH group and capsaicin group, while there was mesocolic lymph node totally replaced by metastatic cancer tissue in the cold exposure group, and the lymph node metastasis rate was 20.0% (Table 1).

3.2 Alterations in collagens after Cold exposure and Capsaicin Treatment

Colon tissue sections were stained by Masson’s trichrome and picrosirius red to identified the total collagens in the colon mucosa. As shown in Fig.3A, there were few collagen fibers in normal colonic mucosa. after DMH treatment, wave shape collagens stained blue were markedly increased around the glands and that this increased further in the cold exposure and capsaicin treatment group. The collagen density was quantified using ImageJ, and it was also showed a significant increase in the colonic tissue of cold exposure and capsaicin group. This excessive collagen deposition was further confirmed by the picrosirius red staining. Sirius red staining reveals in normal tissue, the collagen fibers showed a sparse deposition composed of thin collagen fibers. The collagen fibers in the DMH group were denser than normal collagen fibers. In the capsaicin treatment group, collagen fibers showed an evident increase and were crosslinked into bundles. On the other hand, in cold exposure group
apparently displayed an increased amount of collagen fibers with heterogeneous thickness and alignment. The collagen in cold exposure and capsaicin group were exhibited a predominant reddish or yellow-orange. The structure and organization of collagen fibers were evaluated in colon tissue sections by quantifying the polarization microscopy (PSL-POL) images. Visualized collagen fibers were extracted and analyzed for fiber width, angle, length, and straightness using CT-FIRE software. As shown in Fig. 3B, compare with the DMH group, collagen fibers in the cold exposure and capsaicin group showed a significant increase in angle, length, width, and straightness. These results revealed that cold exposure and capsaicin induced a progressive increase in the content and orientation of collagen fibers in CRC as a function of malignant.

3.3 Alterations in elastin after Cold exposure and Capsaicin Treatment

Weigert’s Resorcin Fuchsin was used to identify the elastin fibers which stained black. As shown in Fig. 3A, elastin was hardly expressed in the colonic mucosa of the normal rats. Treated with DMH, the elastin fibers aligned surrounding the epithelium and the stroma. After cold exposure and capsaicin treatment apparently displayed increased amount of elastin fibers, and thick elastic fibers highly disorganized between the gland compared to their respective control groups and DMH groups.

3.4 Alterations in mRNA levels of COL I, COL III, LOXL2, and elastin.

The expression of COL I, COL III, LOXL2, and elastin mRNAs in colonic tissue were shown in Fig. 5. The COL I, LOXL2, and elastin mRNA levels were higher in the DMH-induced cancer group than in the control group. When compared to DMH group, a significant increase was detected both in the cold exposure and capsaicin treatment group, but the mRNA levels of COL III statistical difference between DMH group and capsaicin exposure group was not significant.

3.5 Alterations in Protein levels of COL I, COL III, LOXL2 and elastin.

The expression of COL I, COL III, LOXL2 and elastin in colonic tissue were showed in Fig. 6 A and B. The protein expression levels of collagen type I, III, LOXL2, and elastin were significantly elevated in colonic tissue from DMH-treated rats in comparison to the control group. cold exposure and capsaicin treatment group
were increased expressions of these proteins. COL I and LOXL2 levels were significantly higher in the cold exposure group, although there was no statistical difference in the change of COL III and elastin between cold exposure and capsaicin group.

3.6 Alterations in protein levels of MMP1, MMP2, MMP9, and TIMP1.

The expression of MMP1, MMP2, MMP9, and TIMP1 in colonic tissue were showed in Fig. 7 A and B. Significantly elevated MMP1, MMP2, MMP9, and TIMP1 immunoreactivity were observed in DMH-treated rats compared to the control group. Cold and capsaicin group increased expressions of these proteins relative to expressions observed in DMH group. When compared to the capsaicin group, a significant increase in expression of MMP2, MMP9, and TIMP1 was observed in the cold exposure group.

4 Discussion.

ECM has been increasingly considered as an important regulator at diverse aspects of tumor initiation, promotion, neoplastic transformation, invasion, and metastasis [31]. Furthermore, ECM remodeling has also been recognized as a consequence of or increased risk for malignant transformation of colonic, hepatic, pulmonary, and pancreatic cells [32, 33]. Collagen and elastin are major components of ECM and their excessive deposition has been implicated in a number of diseases, in particular fibrosis and cancer. However, the morphology and structure of collagen and elastin fibers in animal models of CRC remains unclear. In this study, we analysis morphology and structure of collagen and elastin fibers in rat experimental model of 1,2-dimethylhydrazine-induced colorectal cancer imposed by cold and capsaicin exposure has revealed an association between collagen expression or ECM modifying enzymes and CRC development supporting the notion that ECM remodeling is highly relevant to CRC cancer progression.

Tumor tissue often exhibits fibrosis, and this fibrotic state is characterized by excessive deposition of collagen and elastin [34]. Fibrosis can develop in nearly any organ which is an important driver of tissue stiffness and increases the risk of malignancy [35]. In fibrotic kidney biopsy specimens or multiple experimental kidney
fibrosis rodent models, accumulated elastin can be observed in renal tissue [36]. In human fibrosis of the liver, kidney, and pancreatic, the ECM on average becomes stiffer than normal. Our previous study indicated that in human CRC the collagen development features numerous changes in composition and organization when compared to the normal colonic tissues [37]. In this study, we found that collagen components were quantitatively and qualitatively changed in the rat experimental model of CRC. Using picrosirius red, Masson’s trichrome, and Weigert’s Resorcin-Fuschin staining, we revealed that a marked increase in collagen and elastin deposition was observed in rat exposure of cold and capsaicin. Furthermore, they exhibited more orderly organized based on collagen fibers being more aligned with each other, longer, wider, and being slightly straighter. It has been reported that the structure, orientation and physical properties of the collagen regulate the aggressive behaviour of cancer. For example, Glioblastoma, Kelli B. Pointer, et al. showed that patients with more organized GBM collagen survive longer than patients with less organized GBM collagen [38]. In another work, Zhi-Hua Zhou also demonstrated that increased density, length or width of collagen negatively impact patient of gastric cancer prognosis [39]. The stromal tissue in CRC has also shown that increase in the collagen content of the ECM increasing mechanical stiffness which predisposing to aggressive CRC [40]. In human breast cancer, linear organization and relatively straight collagens that facilitates migration of tumor cells are indicative of worse cancer outcome [41]. Similarly, our data indicated that cold and capsaicin exposure increased collagen and elastin deposition, triggering alterations in the ECM architecture and organization in the DMH-induced CRC for further tumor development and progression. Furthermore, all parameters of collagen fibers (density, angle, length, width, and straightness) were significantly increased in the cold exposure group and could explain the cold-induced CRC more seriously. More importantly, the results suggest a need for future exploration of the tumor-associated ECM influence CRC progression.

Recently, the relationship between ECM remodeling and malignant transformation of cancer has attracted much attention [42]. COL I is the most
abundant protein present in the body. It has been reported that COL I, a major component of collagen, was significant up-regulation in CRC tissues and its shows enhanced CRC migratory capabilities through overexpression of WNT/PCP signaling pathway [43]. Another report pointed out that elevated expression of type I collagen in CRC tissues is correlated to patients with high metastasis which was due to the activation of PI3K/AKT signal [44]. M. K. Bode et al also showed that an expression of COL I was clearly increased in malignant colon tissue in comparison with COL III [45]. Moreover, the elevated expression of COL I has been linked to the invasive and aggressive behavior of CRC [46]. In this study, we also evaluated the expression of COL I in cold exposure and capsaicin treatment CRC colonic tissue. The increase in COL I expressions in our study is consistent with other reports in CRC. Although, the mRNA level of COL III was no significant difference between DMH and capsaicin group. In addition, compared with other groups, the COLI/ COL III in cold exposure group was also significantly increased. With the increasing collagen expression, distribution area, and collagen ratio, aggravating the degree of fibrosis in ECM pathological characteristics will be aggravated [47, 48]. Therefore, the colonic tissue stiffness was significantly higher than that of the other groups and the degree of ECM fibrosis in CRC with cold exposure is more serious than that in other groups.

ECM remodeling is regulated by extracellular matrix enzymes such as LOXL2, MMPs, TIMPs. Research has shown that ECM-crosslinking enzyme LOXL2 has been implicated in stiffness-associated tumor progression [49]. The LOXL2-mediated collagen cross-linking, in in vitro and in vivo models of CRC results in increased tissue stiffness and activation of the FAK/SRC signaling [50]. MMP1, MMP-2, and MMP-9 play a fundamental role in many pathophysiological processes such as cell migration, angiogenesis, and the invasion and metastasis of malignant tumors [51]. TIMPs is the most important physiological inhibitors of MMP, which are also commonly expressed in tumor sites [52]. It was reported that the expression of both MMP-2 MMP9 and TIMP-2 was higher in invasive tumors, and was strongly associated with angiogenesis in DMH-induced CRC [24]. Previous study indicated that cross-linking of collagen is known to activate enzymes involved in matrix
remodelings, such as LOXL2, MMPs, and TIMPs [53, 54]. Although roles of MMPs responsible for the degradation of ECM, LOXL2 mediate ECM cross-linking and stiffening [55]. However, recent studies indicated that LOXL2 activity promotes breast cancer metastasis by regulating the expression of MMPs and TIMPs involved in matrix remodeling [56]. LOXL2, TIMP1, and MMP9 were coexpressed during mammary metastasis, suggesting they function together in glandular remodeling. Our previous study also found that expression of LOXL2, MMP1, MMP2, and MMP9 are positively correlated in colorectal cancer tissues, and they play synergistic roles in ECM remodeling of human colorectal cancer [37]. In the present study, it was analyzed LOXL2, MMP1, MMP2, MMP9 and TIMP parameters and indicated that there was significantly increased in the expression of these proteins in cold exposure and capsaicin group which was accompanied by enhancement of collagen and elastin deposition, and it likely they may act together regulate ECM remodeling.

Conclusion
In summary, the present study unraveled profound remodeling of the ECM in the cold exposure and long-term administration of capsaicin at a low dose treatment rats. Collagen signatures including angle, length, width, and straightness have a great impact on CRC progression. Additionally, our results show that higher colonic tissue stiffness might be rise from extracellular matrix enzymes-mediatesed ECM crosslinking and excessive deposition of collagen and elastin and that such changes are strongly associated with the tumor progression of cold and capsaicin exposure CRC. A better understanding the role of extracellular matrix remodeling and extracellular matrix enzymes on the pathogenic mechanisms of colon cancer may help to underly the molecular mechanism of CRC progression which could afford a novel therapeutic intervention in the treatment of this disease.

Conflicts of interest
The author declares that there are no conflicts of interest.

Author’s Contributions
Jingchun Qin and Weitao Yu mainly takes charge of writing and researching. Bin
Wen designed the study. Jingchun Qin, Weitao Yu, Huixuan Li, and Yuqi liang conducted the experiment and analyzed the data. Feifei Nong contributed to review and edit the paper. Bin Wen revised the manuscript.

**Foundings**

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Graphical abstract:

Cold exposure

Capsaicin

DMH-induced CRC Rat

Compliant

Stiffness

ECM Remodeling

Colonic epithelium cell

Basement membrane

Interstitial matrix

↑ Collagen and elastin deposition

↑ LOXL2, MMPs, and TIMP1 expression

Stiffness

CRC Progression
Legends of Figures

Fig. 1. Changes in colon length and colonic morphology (A, B). Stiff colonic tissues in cold exposure group (C).

Fig. 2. A. Macroscopic image of the colonic tumors. B. Representative sections stained with H&E showing the histopathology of the Mesocolic lymph node in the different groups. C. Representative sections stained with H&E showing the histopathology of the colonic mucosa in the different groups. (A) Normal architecture of colon was observed in the control groups. (B) adenoma with mild dysplasia with
massive infiltration of inflammatory cells. (C) Histology of adenoma with moderate dysplasia in cold exposure groups. (D) Histology of adenoma with severe dysplasia. (E) Histology of well-differentiated tubular adenocarcinomas. (F) Histology of Moderately differentiated adenocarcinomas. (G) Histology of Poorly differentiated adenocarcinomas. (H) Histology of Mucinous adenocarcinoma with signet ring cells (H&E staining, x400, scalar bar 20um).
Table 1 Incidence of various tumors induced in different treatment groups

| Group          | Total number | Adenoma incidence (%) | Adenocarcinoma incidence (%) | With lyphytic metastasis (%) |
|----------------|--------------|------------------------|------------------------------|------------------------------|
|                | of tumors    | Mild                   | moderate                     | Severe                       | Well-differentiated          | Moderate-differentiated      | Poor-differentiated         | Mucinous                    | Metastasis rate |
| Control        | -            | -                      | -                            | -                            | -                            | -                            | -                            | -                            | -              |
| DMH            | 23           | 2/23 (8.7%)            | 2/23 (8.7%)                  | 3/23 (13%)                   | 12/23 (52.2%)                | 4/23 (17.4%)                | 3/23 (13.0%)                | -                            | -              |
| Cold exposure  | 38           | 1/38 (2.6%)            | 2/38 (5.3%)                  | 3/38 (7.8%)                  | 5/38 (15.8%) **              | 8/38 (21.1%)                | 15/38 (36.8%) *             | 4/38 (10.5%)                 | 20.0%          |
| Capsaicin      | 34           | 2/31 (5.9%)            | 1/34 (2.9%)                  | 4/31 (12.9%)                 | 7/31(22.6%) *                | 12/31 (38.7%)               | 7/31(22.6%)                 | 1/31 (3.2%)                  | -              |

Values are expressed as the proportion of lesions-bearing rats. N=10 rats/group. Incidence data was analyzed by using chi-square or Fisher’ exact test. * DMH compared with Cold exposure and Capsaicin-treated group. * p < 0.05, ** p < 0.01.
Fig. 3. Changes in ECM components (collagen fibers and elastin) in colonic mucosa of different treatment groups. (A) Representative photographs of colonic tissues in rats of normal, DMH, cold exposure and capsaicin groups using Masson’s trichrome: collagen (blue), nuclei and cytoplasm (red); picrosirius red in bright-field: collagen (red); polarized light: collagen (yellow-orange to green birefringence) and Weigert’s Resorcin-Fuschin: elastin (blue-black), myofibers (yellow). Magnification, x400, scalar bar 20um. (B) Quantitative analysis of picrosirius red staining, trichrome and weigert’s staining as a measure of collagen and elastin density.
**Fig. 4.** Collagen fibers were automatically extracted for analysis using open-source software CT-FIRE. Then, histograms were generated to show the distribution of various parameters in each polarized light microscopy imaging (A). Quantitative analysis of collagen fibers from polarized light microscopy imaging in the colonic mucosa of different treatment groups (B). Data are mean ± S.E. of three images per tissues region. Comparisons: # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group. # p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&&p < 0.01.
Fig. 5. The mRNA expression levels of Collagen type I, III, elastin and LOXL2 in the colon tissues of rats in different groups. Data were presented as the mean ± S.D form six independent experiments. Comparisons: # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group. # p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.
Fig. 6. Changes in collagen, elastin and LOXL2 proteins in the colonic tissues of different treatment groups. (A). Representative images of immunohistochemical staining (IHC) analysis of COL I, COLIII, LOXL2 and Elastin in the colonic tissues via immunohistochemical staining. Magnification, x400, scalar bar 20um. The data represented IOD SUM/Area SUM. Comparisons: # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group. # p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.
Fig. 7. Changes in MMP1, MMP2, MMP9 and TIMP1 proteins in the colonic tissues of different treatment groups. (A). Representative images of immunohistochemical staining (IHC) analysis of MMP1, MMP2, MMP9 and TIMP1 in the colonic tissues. Magnification, x400, scalar bar 20um. The data represented IOD SUM/Area SUM. Comparisons: # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group. # p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.
### Table 2  COL I, COL III, LOXL2 and Elastin immunoreactivity (IOD)

| Group         | Col I       | COL III     | LOXL2       | Elastin     |
|---------------|-------------|-------------|-------------|-------------|
| Control       | 0.0115±0.073 | 0.0039±0.0028 | 0.0188±0.0073 | 0.0045±0.0035 |
| DMH           | 0.070±0.0137 # | 0.0237±0.1156 | 0.0448±0.0088 # | 0.0512±0.0070 # |
| Cold exposure | 0.1926±0.2689**&| 0.0736±0.0156** | 0.1258±0.1681**& | 0.1048±0.0326* |
| Capsaicin     | 0.1164±0.2946* | 0.0503±0.0181* | 0.0736±0.0147* | 0.0719±0.0214 |

Data are expressed as mean ± SD. # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group.

# p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.

### Table 3  COL I, COL III and COL I/COL III Area

| Group       | COL I       | COL III     | COL I/COL III |
|-------------|-------------|-------------|---------------|
| Control     | 15188.00±3074.48 | 12486.89±3396.75 | 1.23±0.12     |
| DMH         | 90602.33±19842.18 # | 40265.67±9147.60 # | 2.29±0.48 #   |
| Cold exposure | 345646.78±66112.99**& | 79402.11±13410.59** & | 4.34±0.16**&  |
| Capsaicin   | 171893.44±20298.43* | 59138.67±15567.93 | 3.01±0.67*    |

Data are expressed as mean ± SD. # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group.

# p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.

### Table 4  MMP 1, MMP 2, MMP 9 and TIMP 1 immunoreactivity (IOD)

| Group         | MMP 1       | MMP 2       | MMP 9       | TIMP 1       |
|---------------|-------------|-------------|-------------|-------------|
| Control       | 0.0038±0.0026 | 0.0055±0.0036 | 0.0089±0.0064 | 0.0054±0.0038 |
| DMH           | 0.0367±0.0094 # | 0.0688±0.0160 ## | 0.0744±0.0251 ## | 0.0701±0.0211 # |
| Cold exposure | 0.0903±0.0163** | 0.1481±0.0103**& | 0.1880±0.0257**& | 0.1928±0.0295** & |
| Capsaicin     | 0.0685±0.0186* | 0.118±0.0123** | 0.1216±0.0224* | 0.1321±0.0322* |

Data are expressed as mean ± SD. # Control compared with DMH, * DMH compared with Cold exposure and Capsaicin-treated group. & Cold exposure compared with Capsaicin-treated group.

# p < 0.05, ## p < 0.01, * p < 0.05, ** p < 0.01, & p < 0.05, &&p < 0.01.
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