

# Mitigative effects of dehydrodiisoeugenol on enteritis and co-occurring dysmotility in murine model

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**Abstract:** The actions and mechanisms of dehydrodiisoeugenol (DEH) on releasing clinical symptoms such as diarrhea caused by inflammatory bowel diseases or colorectal cancer is still unclear. The main purpose is to reveal the mechanism and describe the impacts of DEH on enteritis and accompanying intestinal dysmotility in murine model. The animal model of diarrhea was established through being given acetic acid by intracolonic instillation and restraint stress and the weight of the diarrhea mouse, diarrhea index (the product of stool rate and stool grade) evaluation and then, myeloperoxidase (MPO) activity were determined after administrated with DEH. Meanwhile, the expression of myosin light chain kinase (MLCK) was research by WB method. Moreover, the isolated jejunal segment (IJS) of rats was separated from the intact jejunum and the contractility was measured through BL-420F physiological recording system. DEH could significantly inhibit the intestinal transit in normal mice or diarrhea-predominated mice and reduce the diarrhea index and the level of MPO in mice. DEH concentration-dependently inhibited motility of IJS in different states. DEH significantly markedly ameliorated the histopathology condition and reduce the MLCK expression in acetic acid induced diarrhea mice. DEH simultaneously improved enteritis and co-occurring dysmotility in diarrhea mice characterized by reducing the contractility and MLCK contents in acetic acid induced diarrhea mice.

**Keywords:** Dehydrodiisoeugenol (DEH), enteritis, acetic acid induced diarrhea, dysmotility, myosin light chain kinase (MLCK).

## INTRODUCTION

*Myristicafragrans* Houtt (Myristicaceae) is a common food flavoring in food industry, as well as being manufactured into perfume in beauty processing plant and now its seeds (nutmeg) are widely processed into medicine, because researchers have found they are effective on anti-inflammation, anti-bacterial, angiogenic inhibition etc. (Zhang *et al.*, 2021; Li *et al.*, 2020; Li *et al.*, 2021). Several findings have suggested physiological roles for Dehydrodiisoeugenol (DEH, CAS No. 2680-81-1, fig.1A) which was isolated from nutmeg, in the action of hepatic protection, anti-thrombosis, anti-allergy, anti-oxidation and remedying neoplasm (Li *et al.*, 2020; Li *et al.*, 2021). The severe inflammation can be mitigated to some extent by DEH and it is possibly to add DEH into anti-inflammatory agent (Murakami *et al.*, 2005). However, the effects and the mechanisms of DEH on releasing clinical symptoms such as diarrhea caused by inflammatory bowel diseases or colorectal cancer is still unclear.

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are two prominent types of intestinal diseases which highly negatively affect the life qualities of patients suffering from abdominal pain, dyspepsia, diarrhea, fecal incontinence and so on (Barros *et al.*, 2019; Ng *et al.*, 2018). There is no doubt that recurring and

bloody diarrhea is the most common and debilitating symptom to the patients suffering from IBD (Anbazhagan *et al.*, 2018). Also, there is another main subtype in IBS called IBS with prominent diarrhea (IBS-D) (Pimentel M, 2018). Meanwhile, the characterization of IBD and IBS are an overlap with inflammation as well as co-occurrence of intestinal dysmotility in various situation (Żorniak *et al.*, 2015; Kuo *et al.*, 2015; Radovanovic-Dinic *et al.*, 2018).

Clinical studies and epidemiological investigations have shown that, the inflammation of IBD spreads in digestive system ranging from the jejunum to the rectum (Kim *et al.*, 2012; Vicario *et al.*, 2009). As the above observations suggested, the essential therapy is aimed at improvement of enteritis and co-occurring dysmotility. Whereas rare relative reports, there is not given enough experience on using commonly available drug in clinic. Our previous experimental results found that, DEH could relieve enteritis and modulate beneficially on intestinal contraction in rats. The main purpose of this study is to explore the mechanism and the impacts of DEH on enteritis and accompanying intestinal dyskinesia in murine model.

## MATERIALS AND METHODS

### *Animals and drugs*

All research procedures were conducted in accordance with the rules of animal care. The animal protection

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committee of Liaoning University of Traditional Chinese Medicine authorized funds and laboratory certificated with No. SYXK (Liao) 2013-0009.

The experiment was carried out by using Kunming strain mice, 18 ~ 22 g and Sprague-Dawley (SD) rats, 180 ~ 220 g, provided by Liaoning Changsheng Biotechnology Co., Ltd. with the certification No. SCXK (Liao) 2015-0001. Every five rats were housed into a cage at a controlled temperature and in a 12h light/12h dark cycle with adequate water and food for daily life.

Dehydrodiisoegenol (> 98% pure, free of endotoxin) was brought from Beijing Solarbio Science and Technology Co., Ltd. (Beijing, China). Phenylmethylsulfonyl fluoride (PMSF) was acquired from Sigma-Aldrich (St. Louis, MO, USA). All other chemical reagents were of analytical quality and commercially accessible. Other chemical reagents were able to be available analytical grade and could be prepared with different solution as a proper proportion. The concentration of each component in Krebs bicarbonate buffer is: (in mmol/L): sodium dehydrogenate phosphate 1.8, potassium chloride 4.7, calcium chloride 2.5, magnesium chloride 1.2, glucose 11.5, sodium bicarbonate 18.0, sodium chloride 114.0; pH 7.4.

#### **Experimental design**

We created four groups including a normal control (NC) group and three DEH groups with high, middle and low dose (60, 30, 15mg/kg body mass), with 10 mice in each group. Different doses of DEH was intragastric administration for high, middle and low dose group mice every day, respectively and NC group mice were administered by gavage with the same volume of distilled water concurrently. Continuous administration for 14 days and after that the mice would be offered adequate water to drink without limit in 12 hours. After administration with the black semisolid paste (test meal) given to each mouse, the method of measuring was refer to the previous description (Francis *et al.*, 1997; Francis *et al.*, 1995; Trudel *et al.*, 2002).

On the base of the previous reports, the animal model of diarrhea was established after a slight modification. The first step is to anesthetize animals lightly. And then animals would have an intracolonic instillation and a restraint stress so that in this situation, we provided 0.2 ml 4.0% (V/V) acetic acid to induce diarrhea (La *et al.*, 2003; Yu *et al.*, 2017). The mice in controlled group were given saline by intracolonic instillation.

The successful establishment of diarrhea mice was affirmed by the evaluation of intestinal inflammation degree and intestinal transit. Divide fifty animals into five groups randomly. So except for ten mice in model control group (MC), the others were operated the same as normal mice experiments described above. The administration

method was the same as that in the previous paragraph. The mice in DEH group were orally administered with DEH respectively. Moreover, the NC and MC group mice were given the same volume of distilled water. Mice were fasted for 12 hours with free drinking water after 14 days of therapy. Next, all mice were individually placed in appropriate clear cages and given test meals, after which measurements were taken using the methods outlined above.

In addition, colon segments were dissected from diarrhea mice (MC group), DEH-treated diarrhea mice and NC group. After the isolated intestinal segments were flushed thoroughly, they were prepared to determine the contents of myosin light chain kinase (MLCK) by WB.

#### **Contractility determination in isolated jejunal segment (IJS) of rats**

The method used for the separation of IJS have been described previously (Xiong *et al.*, 2013; Xu *et al.*, 2019) and the contraction amplitude was calculated by BL-420F physiological recording system, Chengdu Techman Software Co. LTD., China. Through the changes of contraction curve after administration, the differences before and after drug treatment were compared. The independent experiment was repeated six times.

#### **Staining process of HE**

To assess the histopathological damage degree of colonic segments by using light microscope. The mice were decapitated and the colon tissues in different groups were taken off. Then they were cleansed with 4% formaldehyde. These tissues were dehydrated with grade ethanol and then embedded in paraffin followed by hematoxylin-eosin staining. The degree of inflammation and histological damage was observed according to the scoring criteria mentioned previously (Gu *et al.*, 2017; Neurath *et al.*, 1995).

#### **Immunohistochemistry (IHC) of MLCK expression**

Isolated colon tissues were deparaffinized by xylene and then they were hydrated through a series of washes in gradient ethanol and water. Tissue segments were rinsed three times with PBS and each time was lasting for five minutes, before a 30-minutes incubation under 0.1% Tritonx-100. After 3 washes, the samples were then incubated for 30 minutes in confining liquid (5% BSA and 10% sheep serum), followed by bounding primary antibody MLCK (No. 76092, abcam (Hong Kong) Ltd., UK).

They were place in a wet box and the environment temperature was kept at 4 degree centigrade passing the night. The next day, the colonic sections were rinsed with PBS for 3 times (5 minutes per time). The streptavidin protein which was tagged by horseradish peroxidase (HRP) was added dropwise for 40 minutes at 37°C. After washes in PBS for three times, tissues could be stained by

DAB and captured by microscope (Gao *et al.*, 2019). The immunohistochemical staining was performed semi quantitatively by Image J (NIH, Bethesda, Maryland, USA) (Jesen EC, 2013; Blatt *et al.*, 2004).

#### **Western blot analysis of MLCK expression**

Western blotting was carried out as the previous description to examine the expression of MLCK protein from inflammatory colon tissues (Xiong *et al.*, 2016; Xu *et al.*, 2017). Total protein from colon was extracted by a Total Protein Extraction Kit (Nanjing Jiancheng Bioengineering Institute, Lot No. 20201127) following the manufacturer's instruction. After the incubation of Anti-MLCK mAb (1:1000 dilution) kept at 4°C overnight, they were incubated with HRP-conjugated goat antibody against rabbit IgG which has been diluted 1000 times and stained with an enhanced DAB chromogenic kit (Nanjing Jiancheng Bioengineering Institute, Lot No. 20210515). Six independent experiments were carried out and the results were reproducible. The band could be visualized and quantified using Image J Software.

### **STATISTICAL ANALYSIS**

The data is presented as means  $\pm$  standard deviation ( $X \pm s$ ). Statistical methods of one-way ANOVA were used to analysis the difference by the IBM SPSS 21 Statistics software (SPSS Inc., Chicago, IL, USA). And the statistical significance ( $P < 0.05$ ) was regarded to be credible.

### **RESULTS**

#### ***Effects of dehydrodiisoeugenol on the intestinal movement and inflammation in diarrhea mice***

In preclinical studies, the animal weight was recorded daily. In addition, the diarrhea index including the product of stool rate and stool grade and myeloperoxidase (MPO) activity were also monitored in daily life. In this study, the weight changes, state of mind, activity, appetite, hair luster and bowel movement (e.g. fecal occult blood, stool pattern and defecation frequency) of mice were observed every day.

The animals in NC group shows a quicker intestinal transit than those in the groups with DEH treatment, as seen in fig. 1B. fig. 1C indicated that, diarrhea mice in MC group displayed a faster intestinal transit than those in NC group. Different dose of DEH (15, 30 and 60 mg/kg) resulted in the decrease of intestinal transit speed in diarrhea-predominated mice. That is, obvious differences of inhibiting the intestinal motility in a dependent manner with DEH treatment were showed in the results.

The fig.1D showed that the mice of MC lost weight successively, but following DEH could inhibit the loss of the weight and almost approached to the normal level. The diarrhea index and the activities of MPO of mice of

DEH group significantly reduced in comparison with MC group ( $P < 0.05$ ,  $P < 0.01$ ), as seen fig.1E and fig.1f.

#### ***Dehydrodiisoeugenol inhibited the contraction of IJS***

There are the curves of dose-effects relationship in fig.2, which proved that DEH may influence the contraction of IJS with normal contractile state. DEH inhibited the contraction amplitude of IJS in a dose-dependent manner when the concentration was between 10 to 160  $\mu\text{mol/L}$ , especially in excess of 20  $\mu\text{mol/L}$  ( $P < 0.01$ ).

#### ***Dehydrodiisoeugenol inhibits the contractility of IJS in different shirinkage conditions***

In order to test inhibitory effects guided by DEH, three different groups in high and low contraction states were set up respectively in the experiment. DEH reduced IJS contractility in a high state generated by acetylcholine (ACh), high  $\text{Ca}^{2+}$  and histamine, according to the findings. DEH also showed synergistic effects with low  $\text{Ca}^{2+}$ , adrenaline and atropine for the contraction of low contractile states of IJS.

#### ***Histomorphology in diarrhea mice***

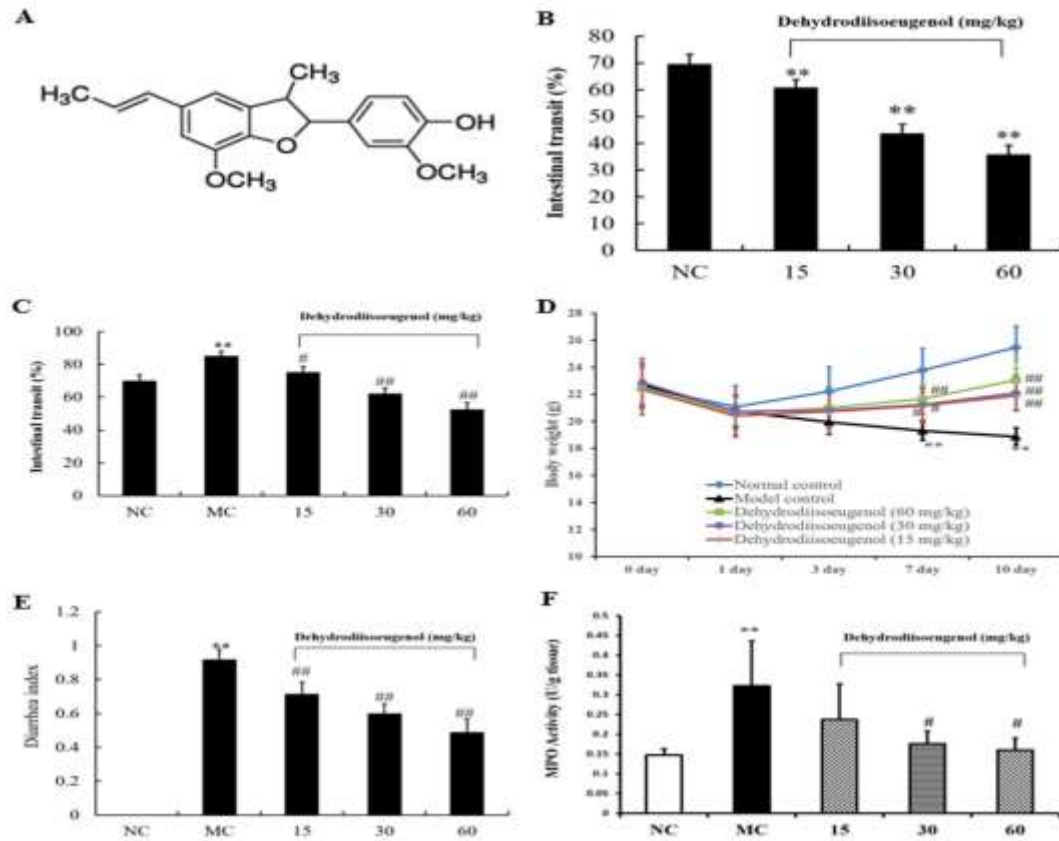
HE staining was used to show whether DEH was able to respite the intestinal injury of diarrhea mice. The histological examination in MC group revealed that, the obvious inflammatory cell infiltration can be seen in the mucosal layer and submucosa, local lymphoid follicles are formed and inflammatory cells invade the local muscle layer, (fig. 4B). In the high-dose group (60mg/kg, fig. 4C), there was a little inflammatory cell infiltration in the mucosal layer, the number of mucosal glands decreased, No inflammatory cell has been detected in the submucosa and muscle layer. In the medium dose group (30mg/kg, fig. 4D), many inflammatory cells were seen in the mucosa and submucosa, no inflammatory cells were seen in the muscle layer and the muscle layer at the inflammatory site became thinner. The results demonstrated that the histopathology condition in acetic acid induced diarrhea mice was significantly ameliorated by DEH treatment. As demonstrated in fig. 4f, the histological assessment of groups administered with DEH (60 and 30mg/kg) were lower than those in MC. DEH resulted in alleviation of inflammation induced by acetic acid by analysis of these changing values.

#### ***MLCK expression in diarrhea mice by IHC***

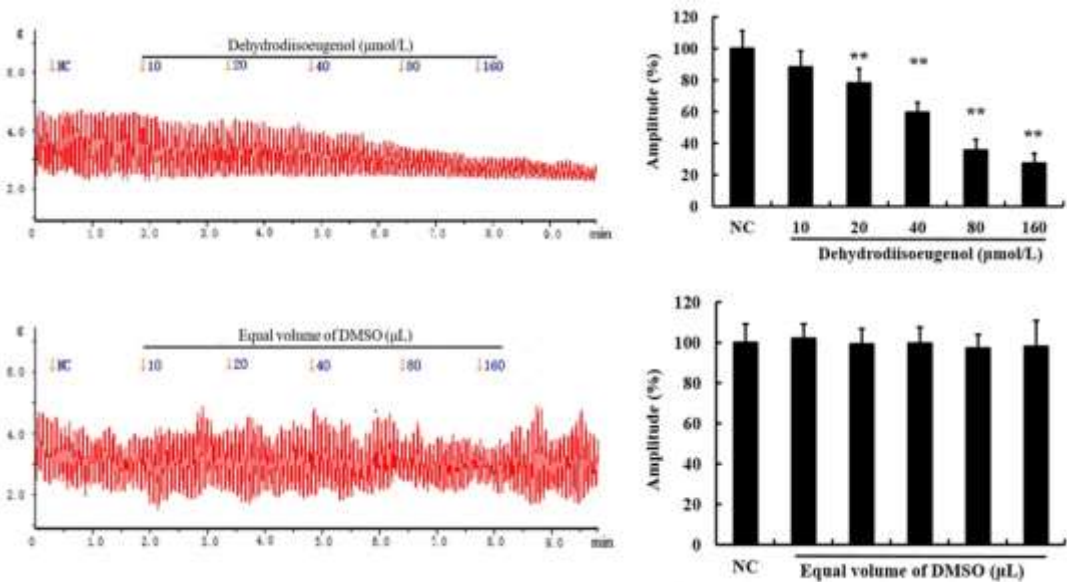
The results of colons MLCK IHC were shown in fig. 5. The MC group had more MLCK expression in the gut than the NC group. In comparison to the MC group, MLCK expression reduced significantly after therapeutic intervention.

#### ***MLCK expression in diarrhea mice by WB***

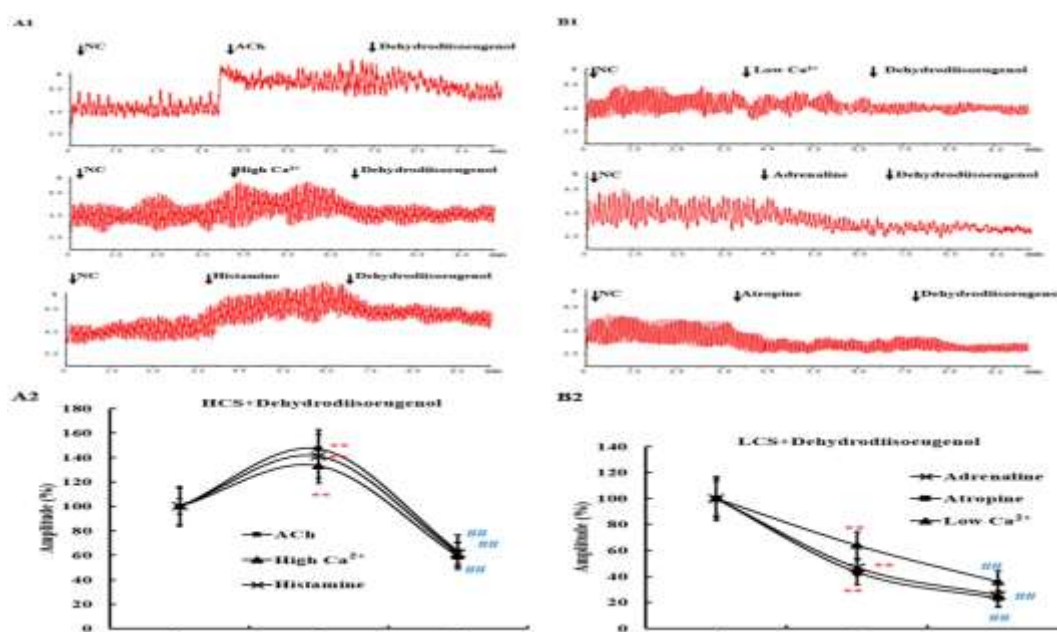
MLCK protein abundance was determined by WB and data were showed that MC group had a higher content of MLCK in comparison with NC group ( $P < 0.01$ ). In the meantime, MLCK expression decreased obviously in the H- and M-dose groups in comparison to MC group (see fig. 6).



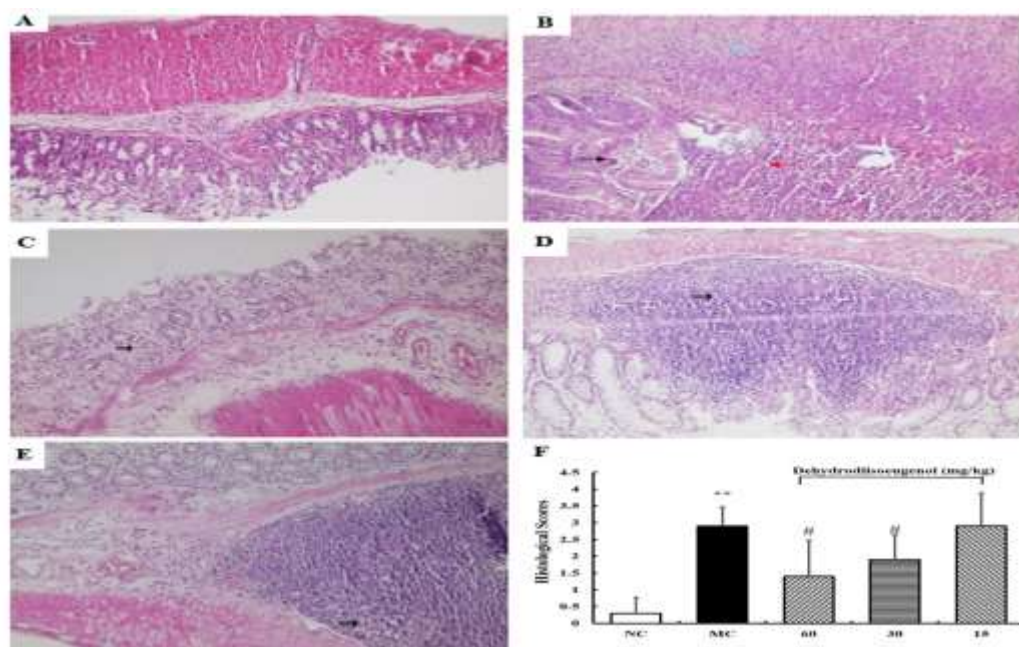
**Fig. 1:** Effects of dehydrodiisoegenol on the intestinal movement and inflammation in diarrhea mice. (A) Exhibiting the structure formula of dehydrodiisoegenol. (B) Exhibiting the action of dehydrodiisoegenol on normal mice intestinal transit. (C) Exhibiting the above action in diarrhea mice. (D) To (F) showing the changes of body weight, diarrhea index and MPO level. \*\* $P < 0.01$  compared to Normal Control (NC), # $P < 0.05$ , ## $P < 0.01$  compared to Model Control.



**Fig. 2:** Dehydrodiisoegenol restrains the contraction of rat isolated jejunal segments. Before the drug treatment, the isolated jejunum tube force contraction was set a relative value to 100% (NC). \*\* $P < 0.01$  compared to NC.

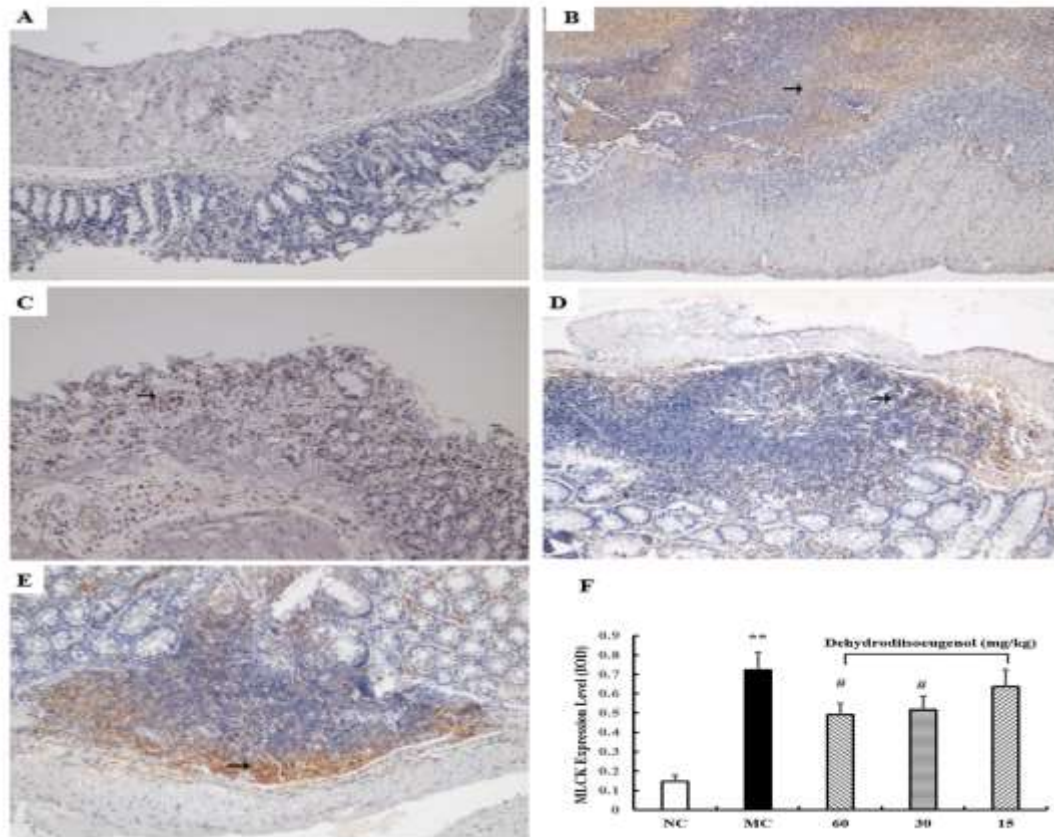


**Fig. 3:** Dehydrodiisoegenol inhibits the contraction of the different contractile states of isolated jejunal segments Side a: Showing the traces (A1) and statistical analysis (A2) of inhibition by DEH on jejunum tube contraction in 3 high shrinkage states. Side B: Showing the traces (B1) and statistical analysis (B2) of inhibition by DEH on jejunum tube contraction in 3 low shrinkage states. The contraction amplitude of jejunum tube in normal shrinkage state is set to relative quantity of 100% (normal control, NC). Other data are relative quantities acquired by comparison with NC. Data are expressed as mean±SD; \*\**P*< 0.01 compared to the NC, ##*P*< 0.01 compared to the contraction amplitudes in high contractile states (HCS) and in low contractile states (LCS) before dehydrodiisoegenol administered respectively.



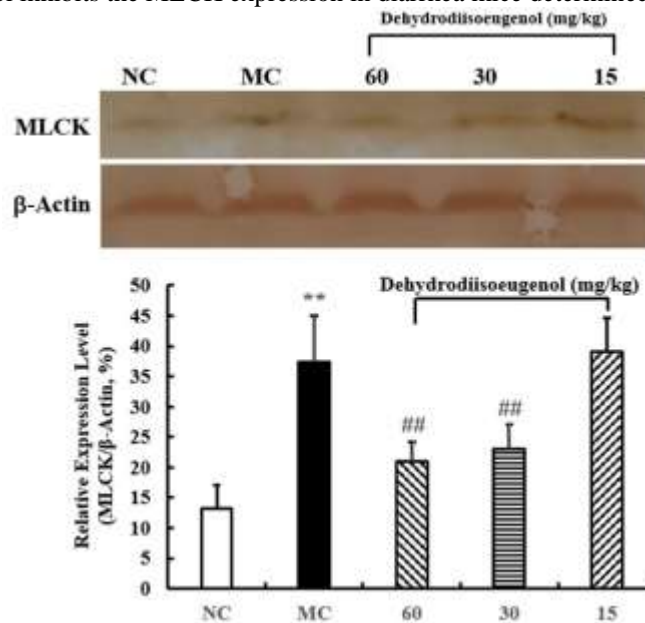
(A) To (E) showing the HE staining of intestine in diarrhea mice treated with dehydrodiisoegenol. (A), Histopathological section in normal control (NC); (B), model control (MC); (C), 80mg/kg dehydrodiisoegenol; (D), 40mg/kg dehydrodiisoegenol; And (E) 20mg/kg. (F) Showing the histopathological evaluation of intestines. \*\**P*< 0.01 compared to NC, #*P*< 0.05 compared to MC.

**Fig. 4:** Histopathological reliefs by Dehydrodiisoegenol in diarrhea mice.



(A) to (E): MLCK expression were determined through IHC ( $\times 100$ ), (A) NC, (B) MC, (C) 60mg/kg dehydrodiisoeugenol group, (D) 30mg/kg dehydrodiisoeugenol group, (E) 15mg/kg dehydrodiisoeugenol group, (F) The semi-quantitative assessment using Image J software.  $**P < 0.01$  in comparison with NC,  $\#P < 0.05$  in comparison with MC.

**Fig. 5:** Dehydrodiisoeugenol inhibits the MLCK expression in diarrhea mice determined through IHC.



**Fig. 6:** Dehydrodiisoeugenol inhibits the MLCK expression in diarrhea mice determined by WB.  $**P < 0.01$  in comparison with NC,  $###P < 0.01$  in comparison with MC.

## DISCUSSION

Inflammatory bowel disease (IBD) including Crohn disease (CD) and ulcerative colitis (UC), is a chronic nonspecific intestinal inflammation (Shivashankar R and Lichtenstein GR 2018). It is widely assumed that MLCK has a significant impact on inflammatory-induced gut barrier disruption stimulated with inflammatory cytokines and the magnitude of MLCK expression and the increasing MLC phosphorylation presence were closely related with active inflammation (Du *et al.*, 2016; Li *et al.*, 2020). Thus, the suppression of MLCK is possibly a potential target in inhibiting inflammatory disease progression. The results indicate that acetic acid-induced diarrhea model mice had a slighter symptom of intestinal inflammation after DEH treatment and the MLCK expression in the intestine was lower than that of diarrhea mice without DEH, which suggest that DEH may ameliorate inflammatory intestine through MLCK pathway.

In this study, DEH dose-dependently reduced IJS motility and restrained the HCS of IJS produced with ACh, elevated  $Ca^{2+}$  and histamine in this investigation. DEH demonstrated a synergistic effect with low  $Ca^{2+}$ , adrenaline and atropine at the same time. In accordance with the inhibition on isolated intestinal contractility, DEH inhibited intestinal propulsion in non-diarrhea or diarrhea mice *in vivo*. Totally, as aforementioned results, DEH definitely could repress the intestinal contraction in mice.

As is known to all, the 20 kDa myosin light chains (MLC<sub>20</sub>) plays a vital role for regulating the smooth muscle shrinkage and  $Ca^{2+}$ /calmodulin dependent MLCK is the main active substance (Gao *et al.*, 2013). Upon stimulation, intracellular calcium release and extracellular calcium ions flow into cells. The increasing concentration of  $Ca^{2+}$  boosts the speed of  $Ca^{2+}$  binding to calmodulin, causing MLCK to activate later. The MLC<sub>20</sub> is phosphorylated by activated MLCK, which leads to the production of contractile force (Lin *et al.*, 2011). Increased MLCK expression may also be possible mechanisms for improved contractions as seen in sensitized airways (Ammit *et al.*, 2000). The loss of myosin phosphorylation was dramatically reduced when MLCK was knocked out, displaying that MLCK is required for smooth muscle shrinkage and gastrointestinal movement *in vivo* (He *et al.*, 2008).

Up to now, IBS, a commonly functional gastrointestinal disorder is still not discovered its etiology and we designed the study based on previous knowledge. In conformity to the inhibition on intestinal motility, DEH down-regulated MLCK expression in intestines of diarrhea mice. These findings suggest that the decrease of MLCK contents is related to DEH-induced inhibitory effects.

To summarize, DEH concurrently ameliorate enteritis and associated abnormal intestinal peristalsis in diarrhea mice characterized by reducing the contractility and MLCK contents in intestines, indicating that DEH may possess latent uses to alleviate the inflammatory pathology of intestine and associated abnormal intestinal peristalsis. Of course, the specific mechanism of the above role of DEH still needs to be further investigation.

## CONCLUSION

Here we provide evidence of inhibition of DEH on isolated intestinal contractility and intestinal propulsion in non-diarrhea or diarrhea mice *in vivo*. Specifically, DEH concurrently ameliorate intestine inflammation and associated abnormal intestinal peristalsis in diarrhea mice possible by MLCK contents in intestines. Of course, additional investigations would be required to research the detailed mechanism of DEH on enteritis and co-occurring dysmotility in murine model.

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