Enhanced efficacy of 5-fluorouracil combined with chrysin in treating colorectal cancer in BALB/c mice: Impact on β-catenin and inducible nitric oxide synthase expression

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Chrysin is a naturally occurring bioflavonoid found in honey and propolis, with anti-cancer and anti-inflammatory properties. This study aimed to investigate the therapeutic effect of chrysin combined with 5-fluorouracil (5-FU) in the treatment of mice with colorectal cancer (CRC). Moreover, the effects of these two compounds on the expression of β-catenin and inducible nitric oxide synthase (iNOS) were investigated. The CRC was induced in the mice by azoxymethane (AOM). The co-administration of 5-FU and chrysin in the treatment of mice reduced the number of aberrant crypt foci (ACF) and the pathologic lesion percentage compared to other treatment groups (P <0.05). The co-administration of 5-FU and chrysin resulted in a reduction in β-catenin and iNOS (P <0.05). We showed that a combination of 5-FU and chrysin is superior to 5-FU or chrysin alone in the treatment of mice with CRC. Our approach opens an avenue to introduce a useful therapeutic option for colorectal cancer in humans.

Keywords: Azoxymethane, Catenins, Chrysin, Colorectal neoplasms, Fluorouracil, Nitric oxide synthase type II

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Introduction

Colorectal cancer (CRC) is the third death leading cancer in the world1-2. Surgery and chemotherapy are frequently used as treatments for CRC. 5-fluorouracil (5-FU) is one of the common drugs in the treatment of CRC and its use in chemotherapy is limited due to corresponding side effects and toxicity3. Herbal compounds are promising candidates for cancer treatment due to their proapoptotic, anti-proliferative and anti-inflammatory potential. Therefore, phytochemicals such as polyphenolic compounds and flavonoids have been exploited to increase the efficacy and reduce the side effects of chemotherapy drugs4,5. Chrysin is a bioflavonoid that is in many plants, honey and propolis6.

In recent years, in-vitro and in-vivo studies have provided additional information about the anti-cancer and anti-inflammatory action of chrysin7-8. Chrysin inhibits cell proliferation in rat C6 glioma cells by increasing p21 protein level9. In another study, chrysin shows an antitumor effect as a result of the inactivation of phosphatidylinositol 3-kinase (PI3K)/Akt signal pathway in U937 cells10 and inhibiting malignant cell growth by downregulated expression of proliferating cell nuclear antigen (PCNA) in HeLa cells11. Chrysin also inhibits the growth of the human lung adenocarcinoma epithelial cell line (A549) by inducing apoptosis12. Despite many studies on the anti-cancer activity of Chrysin, the toxicity of Chrysin has not been reported so far. However, some studies showed that administration of Chrysin to rats for a long period had no toxicity13,14. Wnt/β-catenin and inducible nitric oxide synthase (iNOS)/Nitric oxide (NO) pathways play a key role in carcinogenesis15.

Inflammation is also associated with mutation induction, altered proliferation, and cellular differentiation in the onset and progression of CRC16. An increase in the expression of iNOS has been reported in the CRC-related inflammatory sites17,18. The iNOS generates NO to cause DNA changes, such as base oxidation, deamination, DNA fragmentation, and inhibition of DNA repair19. The increased NO by activating the matrix metalloproteinases eventuate in the decomposition of E-Cadherin and its subsequent separation from β-catenin, thus participating in the cytoplasmic β-catenin accumulation and nuclear β-
catenin. The iNOS also activates β-catenin signaling due to reducing the Wnt/β-catenin regulator Dickkopf-1 (DKK1) transcription. An ideal animal model for evaluating CRC treatment is to use the combination of azoxymethane (AOM) carcinogen and dextran sodium sulfate (DSS) in mice. In this model, in addition to inducing mutations in the β-catenin gene, the iNOS expression increases with chronic inflammation in colonic epithelial cells.

*In-vitro* and *in-vivo* studies have investigated the anti-cancer effects of chrysin. To the best of our knowledge, there are no studies on the anti-cancer effect of chrysin in combination with 5-FU in CRC. Therefore, the present study aimed to evaluate the anti-cancer effects of chrysin combined with 5-FU and to assess the expression of β-catenin and iNOS proteins in the animal model.

**Materials and Methods**

**Animals and ethics**

The study was conducted on 42 male Balb/c mice (Pasteur Institute of Iran) with an average weight of 15-18 g. The animals were kept in a 12:12 h light-dark cycle at 22-25°C with a relative humidity of 45-55% with sufficient and free access to water and food. All of the experimental procedures were in compliance with the Guide for the Care and Use of Laboratory Animals (Eighth Edition, 2011, published by The National Academies Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and were approved by the Research and Ethics Committee of Semnan University of Medical Sciences (IR.SEMUMS.REC.1396.28), Semnan, Iran.

**Chemicals**

First, chrysin (Sigma-Aldrich Company) with a molecular weight of 254.24 was dissolved in Tris buffer (pH=9) and then reached pH=7.4 with 1 N HCl. AOM and DSS (MW= 40 KD) were purchased from Sigma-Aldrich and 5-FU from EBEWE Company (Austria). The primary and secondary antibodies DAB substrate/chromogen were purchased from Abcam Company.

**Experimental protocol and design**

The mice models of cancer were induced by the AOM dissolved in normal saline as an intraperitoneal single dose (10 mg/kg), and subsequently, after a week, followed by the administration of drinking water containing 1.5% DSS for one week. 42 mice were divided randomly into 7 groups (n= 6 mice per group), as follows: Group 1 (Control, CON): receiving normal saline intraperitoneally, Group 2 (AOM): cancer induction with AOM and DSS, Group 3 (AOM/5-FU): cancer-induced mice treated with 5-FU (50 mg/kg), Group 4 (AOM/Ch50): cancer-induced mice treated with chrysin at a dose of 50 mg/kg, Group 5 (AOM/Ch100): cancer-induced mice treated with chrysin at a dose of 100 mg/kg. Group 6 (AOM/5-FU/Ch50): cancer-induced mice treated with 5-FU (50 mg/kg) plus chrysin at a dose of 50 mg/kg, Group 7 (AOM/5-FU/Ch100): cancer-induced mice treated with 5-FU (50 mg/kg) plus chrysin at a dose of 100 mg/kg (Fig. 1).

In the above groups, intraperitoneal therapy was started with 5-FU simultaneously with AOM injection at a dose of 50 mg/Kg once a week. Treatment with chrysin was started at the doses administered intraperitoneally with AOM injection for 8 weeks and 5 days a week. The mice in this project were weighed twice a week during the treatment period and received 0.5-1.0 mL saline intraperitoneally in case of weight loss. Weight loss in this experiment is a surrogate marker for colitis severity.

**Preparation of tissue samples**

After euthanasia of the animals with ether, the intestines were completely dissected. The colon was then washed with normal saline and divided into three equal parts: Proximal, Middle, and Distal. For immunohistochemical and aberrant crypt foci (ACF) studies, sections of the distal colon were fixed in 10% formalin.

**ACF analysis**

A piece of the distal colon was fixed in 10% formalin for 24 h and placed on a slide with a
mucosal surface at the top and stained with 0.1% Toluidine blue for 2 min and then washed in Phosphate buffer (pH=7.2). A stereo microscope (with ×60 magnification) equipped with DP11 digital camera was used to observe and count the ACF.

To distinguish the aberrant crypts from surrounding normal crypts, the following features were used: 1. Large luminal opening, 2. Enlarged size, 3. Thickened epithelium and 4. Crypts larger than surrounding normal crypts. The identified aberrant crypts were categorized as follows: Grade 1 (a focus with an aberrant crypt, ACF1), Grade 2 (a focus with 2 to 3 aberrant crypts, ACF2), Grade 3 (a focus with 4 to 10 aberrant crypts, ACF3) and Grade 4 (a focus with more than 10 aberrant crypts, ACF4).

**Histopathological analysis**

Serial cross-sections were examined after hematoxylin-eosin staining for lesions of hyperplasia, dysplasia and carcinoma. We fixed colon tissue sections in 10% formalin and molded them in paraffin. Five 5-μm serial cross-sections were prepared from each block by the microtome and stained with the hematoxylin-eosin staining method (6 mice per group × 5 sections). The histology of each crypt was examined under a light microscope (Reichert DIASTAR JAVELIN, Cambridge instruments Inc. U.S.A, connected to a digital camera DP11, Sony, Japan) for lesions of hyperplasia, dysplasia and carcinoma.

**β-Catenin and iNOS immunohistochemistry**

Immunohistochemistry staining for the distal colon was performed by an automated tissue processor device on formalin-fixed, paraffin-embedded tissues that were sectioned 4 μm thick and placed on positively charged slides. All slides were deparaffinized by xylene and hydrated using ethanol descent concentrations. Antigen retrieval was performed by placing specimens in the Tris buffer (pH=9) at a temperature of 98°C for 30 min. The slides were then washed with TBS + Twin 20 (3×5 min) and the non-specific binding sites were blocked with 1% BSA + 1% FBS solution in TBS buffer for 2 h at the ambient temperature. Endogenous peroxidase activity was inhibited by 3% H2O2 in TBS buffer for 15 min. The incubation was performed with primary Anti-β-catenin antibody (ab32572) and Anti-iNOS antibody (ab15323) diluted in FBS + BSA buffer in TBS (1: 250 and 1: 100, respectively) at 4°C overnight. Next, the slides were washed in the TBS + Tween20 buffer (3×5 min). The incubation was continued with the secondary antibody (goat anti-rabbit IgG H & L, ab97051) conjugated with Horse Radish Peroxidase (HRP) diluted in TBS + BSA 1% (1: 100) for an hour at the ambient temperature. Afterwards, the slides were incubated with DAB substrate/chromogen (0.1% solution) at ambient temperature for 10 min. The slides were counterstained with Mayer’s hematoxylin for 5–10 min. The stained slides were explored for positive or negative β-catenin (Fig. 2) and iNOS (Fig. 3). A section of the distal colon was used without the addition of the primary antibody as the negative control in any run, and β- Catenin and iNOS respectively from colon and lung tissues were employed as positive controls.

Eight microscopic fields (with an area of 2000 μm²) were randomly explored to count the β-catenin or iNOS-positive cells in each section of the distal colon. The β-catenin or iNOS expression was reported as a percentage of the count of positive staining cells divided by the total count of cells counted by histology images analysis software (Motic Images plus 2.0 ML, Co., LTD, China).

**Statistical analysis**

Data were collected, tabulated and presented as mean±S.E.M. Statistical analysis was conducted by SPSS version 22 software using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Differences were considered significant at P<0.05.

**Results**

**General observations**

All the animals remained alive, and none manifested any significant visible toxicity at the doses of chrysin and 5-FU used in this study. In mice, rectal bleeding and diarrhoea were observed following a full cycle of DSS. There was no significant difference in body weight between the groups during the experiment period (Data not shown).

**Effects of 5-FU, chrysin and their combined treatments on ACF formation**

After staining tissue cross-sections with Toluidine blue, the results indicated that AOM and DSS enhance the number of ACF in the mice colon. The treatment with 5-FU or chrysin alone significantly reduced the number of ACF in the mice colon mucosa compared to the AOM group (P<0.05). The co-administration of 5-FU with each of the 50 and 100
Fig. 2 — Immunohistochemical staining of mice colonic tissue using an anti-β-catenin antibody (brown, arrows) in different groups (n=6). Magnification ×40, all slides were counterstained with hematoxylin. a) Control; b) AOM; c) Positive control; d) AOM/5-FU; e) AOM/Ch50; f) AOM/Ch100; g) AOM/5-FU/Ch50; h) AOM/5-FU; i) Negative control.

Fig. 3 — Immunohistochemical staining of mice colonic tissue using anti-iNOS antibody (brown, arrows) in different groups (n=6). Magnification ×40, all slides were counterstained with hematoxylin. a) Control; b) AOM; c) Positive control; d) AOM/5-FU; e) AOM/Ch50; f) AOM/Ch100; g) AOM/5-FU/Ch50; h) AOM/5-FU/Ch100; i) Negative control.
chrysin doses resulted in a significant reduction in the number of ACF compared to the AOM, AOM/5-FU, AOM/Ch50 and AOM/Ch100 groups ($P <0.05$). The number of ACFs was lower in the AOM/Ch50/5-FU group compared to the AOM/Ch100/5-FU group ($P <0.05$) (Fig. 4).

Effects of 5-FU and chrysin and their combined treatments on the histopathological parameters

Histopathological findings (Table 1) showed that AOM caused the development of hyperplasia, dysplasia and carcinoma in the rat colon. The treatment with 5-FU and chrysin at doses of 50 and 100 reduced the number of lesions compared with the AOM group. The co-administration of 5-FU with chrysin in the treatment of rats was more effective than the use of each of these compounds alone. The treatment of rats with 5-FU co-administered with chrysin (at a dose of 50) had the greatest effect on reducing the number of lesions when compared with other treatment groups.

**Effects of 5-FU, chrysin and their combined treatments on **β**-catenin expression**

As shown in Fig. 5, the immunohistochemistry analysis reveals that the AOM raises the β-catenin expression in comparison with the control group ($P =0.0001$). The treatment with 5-FU resulted in decreased β-catenin levels compared to the AOM group ($P=0.03$). The treatment with any of the 50 and 100 chrysin doses also reduced β-catenin compared to the AOM group, but not significantly. Concomitant use of 5-FU with chrysin at a dose of 50 mg/kg significantly decreased β-catenin levels compared with AOM and AOM/5-FU groups ($P=0.0002$), $*P <0.0001$ compared with the control group; $#P = 0.03$ compared with AOM group; $##P = 0.0002$ compared with AOM and AOM/5-FU groups, $p = 0.0003$ compared with AOM/Ch50 group and $p = 0.003$ compared with AOM/Ch100 group; $** p = 0.0001$ compared with AOM group, $p = 0.02$ compared with AOM/Ch50 group and $p = 0.006$ compared with AOM/Ch100 group.

![Fig. 4](image)

**Table 1 — The effects of 5-FU, chrysin and their combined treatments on histopathologic indexes of the colon tissue**

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Total No. of samples (Sections)</th>
<th>Total lesions No./%</th>
<th>Normal No./%</th>
<th>Hyperplasia No./%</th>
<th>Dysplasia No./%</th>
<th>Carcinoma No./%</th>
<th>Lesion burden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>30</td>
<td>0/0</td>
<td>30/100</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0</td>
</tr>
<tr>
<td>AOM</td>
<td>30</td>
<td>23/76.66</td>
<td>7/23.33</td>
<td>5/16.66</td>
<td>12/40</td>
<td>6/20</td>
<td>3.8</td>
</tr>
<tr>
<td>5FU</td>
<td>30</td>
<td>7/23.33</td>
<td>23/76.66</td>
<td>7/23.33</td>
<td>0/0</td>
<td>0/0</td>
<td>1.17</td>
</tr>
<tr>
<td>Ch50</td>
<td>30</td>
<td>3/26.66</td>
<td>22/73.33</td>
<td>8/26.66</td>
<td>0/0</td>
<td>0/0</td>
<td>1.33</td>
</tr>
<tr>
<td>Ch100</td>
<td>30</td>
<td>12/40.0</td>
<td>18/60.0</td>
<td>6/20.0</td>
<td>3/10.0</td>
<td>3/10.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Ch50+5FU</td>
<td>30</td>
<td>3/10.0</td>
<td>27/90.0</td>
<td>3/10.0</td>
<td>0/0</td>
<td>0/0</td>
<td>0.5</td>
</tr>
<tr>
<td>Ch100+5FU</td>
<td>30</td>
<td>5/16.16</td>
<td>25/83.33</td>
<td>2/6.66</td>
<td>3/10.0</td>
<td>0/0</td>
<td>0.83</td>
</tr>
</tbody>
</table>

Total Lesions: The number and percentage of total mice with lesions. Lesion burden: The ratio of the number and percentage of total lesions in each group to the total number of mice (n = 6). Normal: The number and percentage of samples with normal and healthy status compared to total samples. Hyperplasia, Dysplasia and Carcinoma: The number and percentage of samples with pathologic lesions compared to total samples.
Administration of 5-FU in combination with chrysin at doses of 100 mg/kg reduced the β-catenin protein expression compared to the AOM group ($P = 0.0001$), AOM/Ch50 group ($P = 0.02$) and AOM/Ch100 group ($P = 0.006$).

**Effects of 5-FU, chrysin and their combined treatments on iNOS expression**

The immunohistochemical assays (Fig. 6) indicated that the AOM enhanced the iNOS expression in colonic epithelial cells of mice compared with the control group ($P < 0.001$). The treatment with 5-FU or chrysin alone alleviated the iNOS level compared to the AOM group ($P < 0.001$). The concomitant use of 5-FU with chrysin (at a dose of 50 mg/kg) for the treatment of mice resulted in decreased the iNOS compared to AOM, AOM/5FU, AOM/Ch50 and AOM/Ch100 groups ($P < 0.0001$). Administration of 5-FU in combination with chrysin at a dose of 100 reduced the β-catenin protein expression compared to the AOM group. Concomitant use of 5-FU with chrysin at a dose of 50 mg/kg significantly decreased β-catenin levels compared with AOM/5-FU/Ch100 group ($P = 0.003$).

**Discussion**

Conventional chemotherapy drugs have exhibited side effects, and tumour cells can be resistant to chemotherapy by varying the expression of genes and proteins participating in carcinogenesis. Epidemiological, experimental and clinical studies have shown that anti-inflammatory phytochemicals can inhibit the growth and progression of cancer. Therefore, multiple studies have tried to simultaneously use plant compounds to reduce the side effects of synthetic chemical drugs.

In this study, we used AOM (mutagen) and DSS (pro-inflammatory substance) to induce CRC in mice. This is an ideal animal model with many molecular, pathologic (such as ACF) and clinical features similar to human colorectal cancer. ACFs, which occur about 35 to 45 days after AOM injection, are used as a biomarker in short-term chemotherapy studies. Our study was designed in this animal model to examine the chemopreventive efficacy of chrysin alone and in combination with 5-FU. The chrysin is a flavone from the family of flavonoids that has anti-cancer, anti-angiogenesis, anti-metastatic, anti-oxidant, anti-mutagenic and anti-inflammatory properties.

Our study showed that chrysin is able to inhibit the initiation of ACF formation. In addition, the chrysin plus 5-FU had an additive or synergistic effect on inhibiting the ACF formation. In a study, the oral administration of chrysin in rats inhibited the AOM-induced ACF formation.

In the present study, chrysin showed a synergistic effect with 5-FU and led to a decrease in the lesions of hyperplasia, dysplasia and carcinoma caused by AOM in colon tissue. In the current research, the reduction of pathological lesions in the treatment groups corresponded to a decrease in the number of ACFs. The ACFs developed during the hyperplasia and dysplasia of the epithelial tissue of the colon have a high potential for becoming cancerous.

This study investigated the effect of combination therapy of chrysin and 5-FU on the expression of β-catenin protein. The AOM resulted in an increase in β-catenin protein in animal colon tissue. The treatment of animals with chrysin reduced the expression of β-catenin. The co-administration of 5-FU with chrysin for treatment increased the efficacy of 5-FU and reduced the expression of β-catenin protein. Khan et al., have underlined that the chrysin increased the expression of GSK3B protein and decreased the expression of CK-2 and that the β-
catenin serine phosphorylation (33/37) resulted in its reduction in the cytoplasm and the decline in the Wnt signaling pathway in the rat liver cancer model\(^1\). Polyphenolic compounds through affecting a variety of signaling pathways are capable of inhibiting the growth of cancer cells\(^5\). Wnt is a signaling pathway and plays a central role in the regeneration of the intestinal epithelial cells\(^9\). Naturally, the APC proteins interfere with the phosphorylation and destruction of cytosolic and nuclear β-catenin, preventing accumulation. The APC dysfunction and the β-catenin gene mutation cause β-catenin accumulation in the cytosol and transfer to the nucleus. The β-catenin in the nucleus binds and subsequently activates the transcription of TCF and alters the direction of the interstitial crypt epithelial cells from differentiation towards reproduction\(^3\). The β-catenin binding to the TCF/LEF resulted in an increase in the transcription of the oncogenic target c-MYC, cyclin-D1, and Vascular endothelial growth factor (VEGF) genes and, through its effect on NF-kB, increased the iNOS gene expression\(^1\). Many studies have reported increased iNOS expression in tumour cells, including CRC and during inflammation\(^7\). Flavonoids, by reducing the expression of iNOS, have anti-inflammatory functions and inhibit tumour growth\(^3\). In the present study, AOM and DSS led to an increase in the level of iNOS protein in the animal colon tissue, and the treatment of animals with chrysin decreased its expression. The efficiency of 5-FU increased when 5-FU was used in combination with chrysin to treat, further reducing the expression of iNOS protein. In our previous studies\(^33,34\), chrysin combined with 5-FU led to the reduction of cyclooxygenase-2 expression, and propolis combined with 5-FU reduced the expression of cyclooxygenase-2, nitric oxide and β-catenin proteins.

Some flavonoids, including chrysin, are capable of inhibiting cyclooxygenase-2 and iNOS through the activation of peroxisome proliferator-activated receptor-γ (PPAP-γ), which is why these compounds have anti-cancer and anti-inflammatory properties\(^35\). \textit{In-vivo} and \textit{in-vitro} studies\(^36,37\) have demonstrated that NO and peroxynitrite increase COX-2 and tumour growth, and the iNOS signaling pathway has been suggested as an important target for the prevention and treatment of CRC.

**Conclusion**

Our observation revealed that the chrysin had an additive or synergistic effect with 5-FU. It inhibited the ACF formation in the colonic tissue of the animal model by decreasing the expression of β-catenin and iNOS, and perhaps via other possible mechanisms.

Accordingly, chrysin can be considered a natural compound along with the standard 5-FU drug for the treatment of CRC. However, further studies with different doses of chrysin and 5-FU and human clinical trials are needed to clarify if this flavonoid can be suggested as a possible natural agent for treating human CRC.

**Conflict of interest**

The authors declare no conflict of interest.

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