

Chemoprevention of Induced Colonic Aberrant Crypt Foci in Rats by the Combination of Meloxicam and Grapefruit Juice**

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Abstract

Purpose: To explore the chemopreventive effects of meloxicam, grapefruit juice or the combination against the induction of colonic aberrant crypt foci in the rat model.

Methods: Male albino rats were divided into 5 groups. In group A (positive control), rats received oral dimethylhydrazine, which in Group B, rats received oral meloxicam and dimethylhydrazine. Regarding group C, rats were allowed to drink grapefruit juice, *ad libitum*, then received dimethylhydrazine, and in group D, rats received the combination (meloxicam and grapefruit juice). Group E (negative control), received oral saline only. Blood samples were collected every week from groups B & D for meloxicam analysis. All animals were sacrificed under ether anesthesia on the last day of the 15th week. Colon tissues were removed, cut open longitudinally, rinsed with saline and stained with 0.25% methylene blue and then examined microscopically for the presence of aberrant crypt follicle. Samples containing aberrant crypt foci were sectioned at 4µm thickness and stained with hematoxyline and eosin.

Results: Aberrant Crypt Foci (ACF) are considered as an early neoplastic cell lesions that are characterized by unstable colonic epithelia which encompasses many dysplastic crypts. The data obtained in this study revealed that rats which received meloxicam or the combination (meloxicam and grapefruit juice) resulted in a significant reduction in the incidence of aberrant crypt foci counts compared to the control group. While, rats allowed to only drink grapefruit juice, showed insignificant reduction of aberrant crypt foci count. Furthermore, a significant increase in plasma level of meloxicam was detected in rats that received the combination.

Conclusion: It is concluded that the addition of grapefruit juice to meloxicam in the treatment resulted in a greater chemopreventive activity than meloxicam or grapefruit juice. Furthermore, grapefruit juice resulted in a significant rise of plasma level of meloxicam. This is the first time to report the interaction between meloxicam and grapefruit.

Keywords: Clonic Aberrant Crypt Foci, Meloxican, Grapefruit Juice, Rats.

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Introduction

Cancer chemopreventive agents are considered as non-invasive and non-toxic agents that delay, inhibit, or reverse carcinogenesis. Although many anticancer drugs have been developed, still numbers of mortality and morbidity among cancer patient are high; therefore, it is necessary to implement the golden principle of "prevention is better than cure".¹

Dietary phytochemical constituents have been in focus of many researches for colorectal cancer (CRC) chemoprevention. Many of these constituents demonstrated significant activity in this regard. Grapefruit, for instance, is a rich source of many bioactive compounds that might serve as cancer chemopreventive agents.² The consumption of grapefruit was found to decrease the induction of colonic ACF, through suppressing cell proliferation and elevating apoptosis.³ Also, Curcumin, a natural anti-inflammatory constituent of Curcuma; demonstrated a significant chemopreventive activity when administered initiation or promotion steps of CRC in rats.⁴ It is worth mentioning that dietary changes can prevent as much as 70-80% of CRC cases.⁵ On the other hand, several synthetic drugs were investigated as chemopreventive agents.

Among those, the non-steroidal anti-inflammatory drugs (NSAIDs) were the most effective agents in this respect.⁶

Aberrant Crypt Foci (ACF) are considered an early neoplastic cell lesions that are characterized by unstable colonic epithelia which encompasses many dysplastic crypts.⁷ The sequential analyses of growth morphological characteristics of ACF induced by the known carcinogen 1,2-dimethylhydrazine (DMH) in rats, indicates that ACF are precursor lesions of colorectal cancer induced by chemical agents.⁸ Furthermore, it was reported that ACF induced in rats by DMH in short term (4weeks) correlates well with the development of well-differentiated adenocarcinoma in the medium term (30 weeks).⁹

Also, it was found that ACF assay in animal model is a reasonable, sensitive and specific tool for the measurement of the efficacy of chemopreventive agents.¹⁰

The purpose of this study was to assess the chemopreventive activity of meloxicam, grapefruit juice, and the combination (the first time to be evaluated) of both agents against the development of colonic ACF in male albino rat and to explore any possible interaction among both agents given simultaneously.

Materials and Methods

Animals

Seventy adult albino male rats were obtained from the animal house of the Faculty of Medicine at the University of Jordan. Rats were housed in plastic cages, five rats per cage. The bedding of the cages consisted of sawdust which was changed frequently. Rats were kept at room conditions for 2 weeks for the sake of acclimatization and were provided with rat chow and tap water *ad libitum*.

1, 2-Dimethylhydrazine

Dimethylhydrazine-HCl (DMH), Lot # 508178-034, 99%, purchased from Sigma-Aldrich, Riedst Chemie, Steinheimn, Germany, was used in rats to induce ACF.⁸ DMH were prepared as 10 mg per 1ml in saline solution.

Meloxicam

Meloxicam, preferential cyclooxygenase-2, in a powder form was obtained from The Advanced Pharmaceutical Industries Co. Ltd. (Amman, Jordan). Meloxicam purity was checked by uv spectrum and high performance liquid chromatography. Meloxicam was dissolved in 0.15M NaOH solution, with pH 8.5.¹¹ Meloxicam solutions were prepared weekly as 1mg/ml and stored in the refrigerator at 4°C until it was used.

Grapefruit Juice

Four hundred kg of fresh grapefruit were bought

from a local market in Amman, Jordan; the juice was squeezed out manually and then filtered by a cloth bag to remove fibers. The juice was then packaged in plastic bags (300 ml per bag) and stored in a freezer at -20°C until it was used.

Experimental protocol

Seventy rats were divided into 5 groups and labeled A to E. Each group of A, B, C & D groups consisted of 15 rats, while group E consisted of 10 rats.

Animals were treated according to methods of Furihata et al. and Brown et al.^{12, 13} as summarized in Table (1). The duration of experiments lasted for 105 days started from the first DMH dose. During the experiment, 0.5 ml blood samples were collected weekly from rat's tails of groups B & D, for meloxicam analysis. The blood was collected from rats 24 hours after the administration of the third meloxicam dose. Blood samples were centrifuged and the plasma were separated and stored at -20°C until analysis.

Table 1. Treatment Protocol

Group	Treatment	Treatment days	Duration of the study
A	Dimethylhydrazine (DMH) (20mg/kg)	0,3,6, & 9.	105
B	Meloxicam (1.0mg/kg) + DMH (20mg/kg)	0,1,2, (3 Doses per week for 15 weeks). 3,6,9, & 12.	108
C	Grapefruit juice (GFJ) (<i>ad libitum</i>) + DMH (20 mg/kg)	0 till the end of the experment. 7,10,13, & 16.	112
D	GFJ as in C + Meloxicam (1.0mg/kg) DMH as in C	0 till the end of the experment. 4,5, & 6 (3 Doses per week for 15 weeks). 7,10,13,& 16.	112
E	Saline (0.9% NaCL)	0,3,6, & 9.	105

Microscopic Examination and ACF Counting

All histopathological work was done in the laboratories of Pathology Department under the supervision of the co-author (Dr. M.S.).

Rats were laparotomised under ether anesthesia; the entire colons were removed, opened longitudinally, and flushed with saline. Then, each colon was spread flat between two filter papers.⁷ The colons were fixed in 70% ethanol.³ Glass microscope slides were placed on the top of the filter paper to ensure that the colons remain flat.⁸

After 24 hours, each colon was cut into several pieces of 3-4 cm length and each colon was kept individually in plastic container filled with 70% ethanol. Then, colons tissues were stained with 0.25% solution methylene blue for 30-60 seconds. Then, tissues were rinsed with 70% ethanol to remove excess stain. Tissues were placed on slides with mucosa surface kept up and then examined under light microscope. The light intensity was set to maximum so light can penetrate into the colon tissue. The entire colons were scanned for the presence of ACF. ACF were recognized and counted according to the method developed by Bird and Mclellan.^{7,8}

Histological examination

In order to assess the colonic dysplastic lesion, pieces of colon tissue that contained ACF were sectioned at 4µm thickness and stained with hematoxylin and eosin (H & E). Slides were examined microscopically using X40 or X100.

Meloxicam analysis

Plasma samples were extracted for meloxicam and analyzed using HPLC according to Bata et al.¹¹

Statistical Analysis

Statistical analysis was performed using SPSS software. The results were expressed as mean ± standard deviation. Data set were done first by the one way ANOVA. Then, pair wise comparisons were conducted using Student's t-test. P values less than 0.05 were considered significant.

Results

ACF Counts: Colons were entirely scanned for ACF and were observed in the colonic tissues of all groups except the control. Table (2) presents the data of colonic ACF counts of all rat groups. This data shows a significant reduction ($P < 0.02$) in the incidence of colonic ACF in animals treated with meloxicam (group B). Furthermore, a statistically insignificant reduction ($P > 0.107$) in the incidence of colonic ACF was observed in animals treated with grapefruit juice (group C). Whereas, the treatment of rats with the combination of meloxicam and grapefruit juice (group D) resulted in a greater significant reduction ($P < 0.001$) in the incidence of colonic ACF. at the same time, the difference in the reduction of colonic ACF incidence between groups B and D, were not statistically significant ($P > 0.48$), whereas the difference was statistically significant between groups C and D ($P < 0.033$) Table (2). These findings suggest that meloxicam had a significant greater reduction of colonic ACF incidence than grapefruit juice. It is also suggested that an additive chemopreventive effect had occurred in the combination group (D) compared to the results of meloxicam or GFJ each alone.

Table 2. Aberrant foci count of rat colon and meloxicam plasma level (ug/ml)

Treatment	Group	ACF Count (M ± SD)	Significance*	Meloxicam level (M ± SD)	Significance**
Dimethylhydrazine (DMH)	A	66.0 ± 19.1	—	—	—
DMH + Meloxicam	B	40.0 ± 31.1	P < 0.02	4.37 ± 2.1	P < 0.001
DMH + Grapefruit juice (GFJ)	C	52.0 ± 23.8	P > 0.107	—	—
DMH + Meloxicam + GFJ	D	32.0 ± 22.7	P < 0.001	7.81 ± 4.33	—
Control (saline)	E	0.00	—	—	—

*B vs. A

*C vs. A

*D vs. A

** B vs. D

The histological examination

The histopathology of the colonic tissues which contained colonic ACF revealed marked dysplastic with elongated epithelial cells within the crypts of all lesions (Figure 2). Furthermore, the ACF had flat or elevated appearance and luminal opening of the crypts which were noticeably different from normal crypts. No such features were observed in the colonic tissues of the negative control.

Meloxicam Assay

The mean meloxicam plasma concentration for group B was 4.37 ± 2.09 $\mu\text{g/ml}$, while it was 7.81 ± 4.33 $\mu\text{g/ml}$ for group D. The plasma level of group D was significantly greater ($P < 0.001$) than group B. These data suggest that grapefruit juice may inhibit meloxicam disposition in rats.

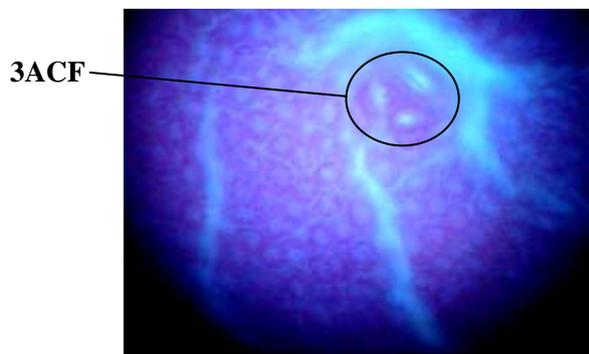


Figure (1): Methylene blue-stained ACF consisting of three colonic aberrant crypt foci induced by Dimethylhydrazine in rat. ACF. $\times 100$.

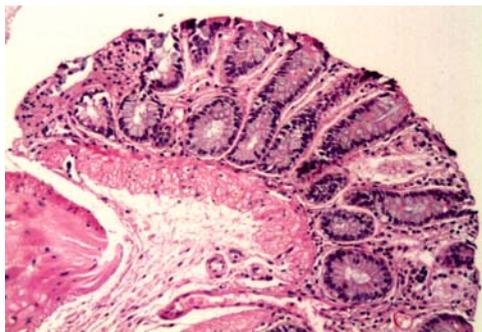


Figure (2): A typical histology of ACF: the dysplastic crypts of the ACF are enlarged and elevated compared with the adjacent normal crypts. ACF stained with H & E. Crypts $\times 200$.

Discussion

The Aberrant Crypt Foci (ACF) is considered an early biomarker lesion for colorectal cancer. In this study, the ACF were induced by the administration of dimethylhydrazine (carcinogen) into rat model. Meloxicam (the preferential COX 2 inhibitor) or grapefruit were used in this study as chemopreventive agents.

In this study, the administration of DMH induced an average of 66.0 ± 19.1 ACF per colon. Meanwhile, the use of DMH and grapefruit juice resulted in a reduction of ACF incidence by 21.2 and 39.4%, respectively. Furthermore, the combination of grapefruit juice and meloxicam resulted in 51.5% reduction of ACF incidence.

Recently, several studies in rodent carcinogen models have been reported; which indicates that NSAIDs were able to reduce the number of ACF.¹⁴⁻¹⁶ Furthermore, it was reported that the dose of NSAIDs was important in ACF inhibition. For example, the supernormal dose of celecoxib, the selective COX-2 inhibitor, resulted in 41% reduction in ACF incidence as compared to 22% reduction when a modest dose was used. In this study, the dose of Meloxicam used, 1mg/kg, is approximately one eightieth of its LD50. Such dose reduced 39.3% of ACF; which is comparable to celecoxib effect.

In a related study done by Brown et al.,¹³ where it studied several NSAIDs agents such as indomethacine, sulindic sulphone, celecoxib, and meloxicam. They found that the chemopreventive efficacy of drugs were independent of cyclooxygenase inhibitor profile.¹³

The use of grapefruit juice in our study resulted in 21.2% in ACF counts. This result is in agreement with Vanamala et al., results except that they found a significant reduction of ACF incidence. We attributed these differences, in part to the different sources and methods of grapefruit preparation.¹³

The histopathological findings of this study, such as the ACF, had flat or elevated appearance, and the luminal opening of the crypts were identical

to shown by other investigators.^{3,9}

This is the first work, as far as we know, to study the combination of meloxicam and grapefruit regarding its chemopreventive effects. This study revealed two important findings. First, a greater significant ACF incidence (51.5%). Second, a significant increase in meloxicam by 78.7% in the level of plasma in rats that received the combination rather than those that received meloxicam. One can assume that this increase in meloxicam plasma level contributed to chemoprevented activity of the combination. The exact mechanism of grapefruit-meloxicam interaction warrants future study.

In conclusion, our results demonstrate a significant chemopreventive activity of the combination. This activity was greater than meloxicam or grapefruit juice. In addition, our results demonstrate a greater plasma level of meloxicam in rats treated with the combination compared to those treated with meloxicam. Grapefruit may thus be useful to be used with meloxicam in the therapy of colon cancer.

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الوقاية الكيماوية لكل من الميلوكسيكام، عصير الجريبفروت و علاج مكون من استخدام كلتا المادتين معا ضد تكون بؤر الخبيثات الشاذة القولونية المحفزة بمادة ٢، ١ - ثنائي ميثيل الهيدرزين في نموذج الجرذان

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الملخص

هدف البحث: دراسة فعالية كل من الميلوكسيكام وعصير الجريبفروت وكذلك فعالية استخدام كلتا المادتين معا في الوقاية الكيماوية ضد تكون
بؤر الخبيثات الشاذة القولونية (ACF) المحفزة بمادة ٢، ١ - ثنائي ميثيل الهايدرزين (DMH)، في ذكور الجرذان البيضاء.

منهجية البحث: سبعون جرذاً تم توزيعها على خمس مجموعات مرقمة A، B، C، D، و E.

المجموعة A: عولجت بأربع جرعات من مادة (DMH) بمقدار 20 ملغم/كغم للجرعة الواحدة.

المجموعة B: عولجت بالميلوكسيكام (1 ملغم/كغم) خلال فترة التجربة، وكذلك عولجت بمادة (DMH).

المجموعة C: تم استبدال ماء الشرب المخصص لها بعصير الجريبفروت واستمر ذلك حتى نهاية التجربة، وعولجت أيضا بمادة (DMH).

المجموعة D: عولجت بالميلوكسيكام بنفس الطريقة المتبعة مع المجموعة B. وكذلك قدم عصير الجريبفروت بنفس الطريقة المتبعة مع المجموعة

C. وعولجت بمادة (DMH).

المجموعة E: عولجت بالمحلول الملحي الطبيعي. خلال التجربة جمعت عينات دم أسبوعياً من المجموعتين B و D لتحليل مستوى

الميلوكسيكام.

بعد مرور 105 أيام على أول جرعة من (DMH) تم الاجهاز على جميع الحيوانات و استؤصلت قولوناتها كاملة وفحصت بالمجهر الضوئي

المركب لاحصاء أعداد الخبيثات (ACF). وبعد ذلك تم عمل شرائح مجهرية من عينات تحوي هذه البؤر

النتائج: كان معدل الـ(ACF) للمجموعات A، B، C، D و E 66 ± 19 ، 40 ± 31 ، $52 \pm 23,8$ ، $32 \pm 22,7$ و $0,00$ على

التوالي. وكان معدل تركيز الميلوكسيكام في بلازما المجموعتين B و D $4,37 \pm 2,1$ و $7,81 \pm 4,3$ ميكروغرام/مل على التوالي.

الاستنتاجات: ان استخدام علاج خليط من الميلوكسيكام وعصير الجريبفروت له فعالية أقوى (جوهرياً) ضد تكون الـ(ACF)، وهذه

الفعالية أكبر من تلك الناتجة عن استعمال أي من هذين العلاجين على حدة. اضافة لذلك فان فعالية الميلوكسيكام في هذا المجال أكبر من

فعالية عصير الجريبفروت.

إن تناول عصير الجريبفروت خلال فترة العلاج بالميلوكسيكام يرفع مستوى الميلوكسيكام في دم الجرذان بشكل ملحوظ.

الكلمات الدالة: الميلوكسيكام، عصير الجريبفروت، بؤر الخبيثات الشاذة القولونية المحفزة، الجرذان.