Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 2, 2014

EFFICACY OF INTRA-PERITONEAL ADMINISTRATION OF METFORMIN AND 5-FLUORURACIL IN PREVENTION OF INDUCED-COLORECTAL ABERRANT CRYPT FOCI IN MICE

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Received: 17 Jan 2014, Revised and Accepted: 29 Feb 2014

ABSTRACT

Objective: The aim of this study is to investigate the anti-proliferative effect of metformin in comparison with 5-fluoruracil (5-FU) in induced-colorectal ACF in mice, and to evaluate the activity of metformin as chemo preventive drug in colorectal cancer when given oral and intra-peritoneal route.

Methods: Sixty albino BALB/c mice were allocated into five groups, the groups given azoxymethane (AOM) as inducer for ACF once weekly for two weeks. Group A treated with normal saline injection and considered as positive control; Group B treated with metformin orally for four weeks, Group C treated with intra-peritoneal (i.p.) metfromin and group D treated with i.p injection of 5-FU. Group E did not receive treatment and consider as negative control. The started one week after the second dose of AOM. The body weight has been measured weekly, while fasting blood sugar (FBS) has been measured at the end of the experiment. Then, all animals were sacrificed and their colon and rectum have been removed for counting the number of ACF and the anti-proliferative effect of metformin and 5-FU on colonic epithelium using proliferating cell nuclear antigen (PCNA) labeling index (LI).

Results: Metformin has the ability to prevent the ACF without causing any change in the level of FBS. Both metformin and 5-FU have significant anti-proliferative activity against proliferation of colonic epithelium. Intraperitoneal administration of metformin has significant chemo preventive effect and anti-proliferative effect than oral administration so as its efficacy become close to that of 5-FU without significant difference between them.

Conclusion: Intra-peritoneal administration of metformin can prevent ACF significantly than oral administration with no change in FBS during the course of the treatment compared to 5-FU.

Keywords*:* Metformin; 5- fluorouracil; Aberrant crypt foci; Chemoprevention**.**

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer, and the fourth most frequent cause of cancer deaths worldwide, with more than 102,900 cases and 51,370 cancer related deaths reported worldwide in 2010 [1]. One way for decrease the incidence of advance CRC and improve the survival of patients is by using chemo prevent agents. Cyclooxygenase-2 (COX-2) is the most effective chemo preventive agents available now a day [2]. For example, the selective COX-2 inhibitor, celecoxib has been approved by American food and drugs administration FDA as an adjunct therapy in familial adenomatous polyposis (FAP) [3]. However, one study mentioned that serious cardiovascular side effects are associated with the using of selective COX-2 inhibitors [4]. metformin is the promising drug which is an anti-diabetic drug [5].

Many studies involving cancer outcomes among diabetic patients receiving metformin therapy have provided preliminary evidence that this compound favorably influences cancer outcomes [6, 7]. Several researches revealed that metformin have ability to prevent many type of cancer. Memmott *et al.*, showed that metformin have the ability to prevent tobacco carcinogen–induced lung tumor genesis in mice [8].

While another experimental study showed that chemo preventive of metformin in azoxymethane –induced colorectal cancer in mice [9]. In addition to that, Goodwin et al. (2009) observed a beneficial effect of metformin in breast cancer[10]. Furthermore, there is another research demonstrated that metformin also inhibits the proliferation of human prostate cancer cells [11].

The aim of the present study was to investigate the antiproliferative effect of metformin in comparing to 5-FU in azoxymethane –induced colorectal ACF in mice and to evaluate the efficacy of metformin as chemopreventive drug in CRC when given by different routes (oral and i.p. route).

MATERIALS AND METHODS

Sixty adult healthy Swiss albino BALB/c mice of both sexes which bred in the animal house of The Center of Quality Control and Drug Research in Baghdad were used. Their ages were six weeks and weighing (18-22gm). They housed in plastic cages containing hard wood chips for bedding. The bedding was changed weekly to ensure a clean environment. The mice maintained in an air-conditioned room at 25±2˚C with 14/10 hour's light/dark cycle. The animals were housed under standard laboratory conditions with food and water provided. The study was approved by the Animal Ethics Committee of Al-Nahrain University / College of Medicine.

The animals were randomized into five groups, twelve mice in each group. Mice of each group (except negative control group) were injected i.p. with azoxymethane (AOM) 10mg/kg (Sigma –Aldrich, St. Louis, MO) once weekly for two weeks then they were treated for four weeks with one of the following drugs starting one week after the second injection of AOM: Group A treated with normal saline (Adwic pharmaceuticals, Egypt) and considered as positive control group; Group B treated with metfromin orally (Wanburg limited, India) dissolved in the drinking water in concentration of 5mg/ml; Group C treated with metformin Intraperitoneal (250mg/kg/ day) in two divided doses; Group D treated with 5-FU I.P. (Edewe pharma, Ruhsat Numarasi) 15 mg/kg/day six times weekly and Group E negative control (did not receive any drug). After 6 weeks all animals were fasted for 16 hours and blood samples were collected from them. About 0.7ml of blood specimens were drawn from the heart of each mouse in all groups to be used for analysis of fasting blood sugar FBS using glucose-TR kit (Spinreact, Spain). After blood collection, mice were sacrificed with an over dose of thiopental sodium 75mg/kg i.p. (Biochem GmbH, Kundl, Austria). Immediately after scarification the colon and rectum (from cecum to anus) were removed, gently flushed with ice-cold phosphate buffer saline (Hi Media laboratories Prt., Ltd.,India) to remove any fecal content then opened longitudinally and fixed flat between two filter papers in 10% neutral buffered formalin. After 24 h in fixative, the samples have been sent to the department of pathology and forensic

medicine at the college of medicine/University of Al-Nahrain to be analyzed by consultant pathologist for identification and counting of ACF. Colons and rectums have been examined individually for visualizing and counting the number of ACF in each mouse. Each colon stained in 0.2% methylene blue (0.1 g in 100 ml distilled water) for 2 min and placed mucosa side up on a glass microscope slide to visualize crypt outlines by light microscope at (40×) magnification.

ACF were distinguished from surrounding normal crypts by increased size, elongated luminal opening, increased distance from luminal to basal surface of cells, containing two or more abnormal crypts with thickened epithelial cell lining, and enlarged pericryptal area relative to surrounding normal crypts [12,13]. After counting the ACFs, dye was removed by 70% methanol with gentle shaking at room temperature for 4–6 min then washed with tap water and dehydrated in different concentrations of alcohol. After that the specimens treated with xylol before dipping in liquid paraffin at 55-60 ˚C and paraffin blocks were made and from this block two sections for each sample were prepared, one on microscopic slide to be stained by hemotoxlyin and eosin (H &E) for hsitopathological examination and the other one was on positive slide for immunohistochemical study to show the antiproliferative effect of metformin and 5-FU on colonic epithelial using proliferating cell nuclear antigen labeling index (PCNA) (Dako,Denmark). The collected data were analyzed statistically using ANOVA test at $P \leq 0.05$ as a lowest hint of significance.

RESULTS

Effect of metformin and 5-FU on the formation of aberrant crypt foci

AOM induced ACF in about 41/48 (85.41%) of AOM-treated mice and no mortality had occurred among them. Table (1) shows highly significant reduction in the total number of ACF in all treated groups (Group B, C and D) ($p < 0.001$) in comparison with group A. Positive control group (Group A) developed 20.42 + 2.08 ACF/mouse. While oral metformin treated group (Group B) developed 9.24+ 0.86 ACF/mouse and this number further reduced to 5.50+0.63 ACF/mouse when metformin administered to the mice intra-peritoneal (Group C). Great reduction in the number had occurred in 5-FU treated mice 1.5 + 0.48 ACF/mouse. In addition to that, there is a significant difference in the reduction of ACF between 5-FU treated mice and oral metformin mice ($p < 0.05$). No significant difference observed between the numbers of ACF counted in the colon and rectum of 5-FU treated mice compared to the number of the ACF counted in i.p. metformin treated mice ($p > 0.05$).

Furthermore, oral metformin therapy showed significant difference ($p < 0.05$) in the number of ACF compared to negative control group (Group E) which did not develop any crypts in the colon of mice and considered as normal group. No significant difference ($p > 0.05$) observed in the number of ACF between Group E and group treated with either metformin i.p. (Group C) or 5-FU (Group D). Histopathological examination of ACF under light microscope demonstrated greater cellular, loss of cell differentiation, reduction in goblet cells and nuclear elongation as showing in figure (2).

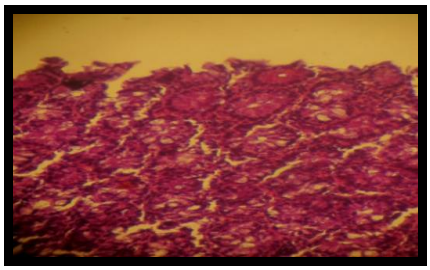

Fig. 1: **Heamatoxylin and eosin staining 10X for colon of AOM-treated mice, notice ACF (arrows) that express greater cellularity, loss of cell differentiation, reduction in goblet cells and nuclear elongation.**

Table 1: The effect of metformin and 5-fluoruoracil on the formation of aberrant crypt foci In colon

Groups	No. of mice	ACF incidence (%)	No. of ACF/mouse [a]	Inhibition of ACF (%)
Group A (Positive control group)	12	12/12 (100%)	20.42 + 2.08	-
Group B (oral metformin group)	12	12/12 (100%)	9.24 + 0.86 *	54.70 %
Group C (I.P metformin group)	12	10/12 (83.3%)	5.50 + 0.63*	73.06 %
Group D (I.P 5-FU group)	12	7/12 (58.33%)	1.50 + 0.48*	92.65 %
Group E (Negative control group)	12	0/12 (0 %)	-	-

[a]Mean + standard error

*differences were highly significant comparing to value in the control group; $P < 0.001$ in comparison with group A

Effect of metformin and 5-FU on proliferation activity of colonic epithelium

The results presented in figure (3) indicated that the PCNA LI of oral metformin treated group, i.p metformin treated group and 5-FU treated group, were significantly lower (20.00+ 17.02, 7.50+8.80 and 2.50+4.18 respectively); ($p < 0.05$) than positive control group (53.00+ 29.43).

Table 2: Fasting blood sugar (mg/ 100ml), in serum of mice after 4 weeks treatment with either metformin or 5-FU comparing with positive and negative control groups.

Group	No. of mice	FBSmg/100ml [a]
Group A (Positive control group)	12	55.83 + 4.54
Group B (oral metformin group)	12	55.33 + 4.49
Group C (I.P metformin group)	12	51.92 + 2.58
Group D (I.P 5-FU group)	12	84.92 + 3.50*
Group E (Negative control)	12	58.17 + 2.00

[a]Mean + standard error

*differences were significant compare with values in the control group; $P < 0.05$ in compassion with group A.

Effect of metformin and 5-FU on the body weight:

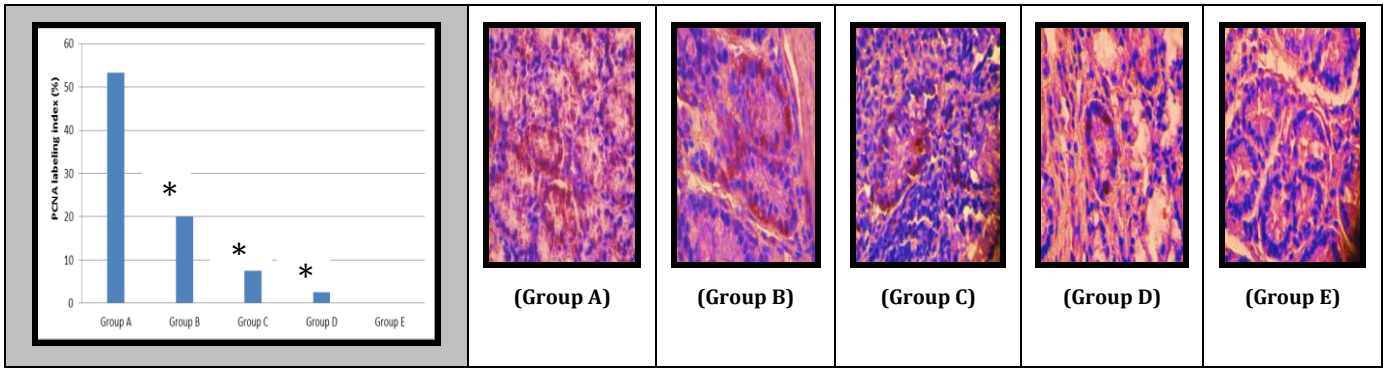

Fig. 2: cell proliferation assay using PCNA labeling index in each group in the experiment expressed as the percentage of positively stained nuclei (brown color) out of the total number of nuclei counted in the crypts of the colon. Each bar represent the mean,* *P* < 0.05. Represntative immunohistochemical staining for PCNA in each group showing positive expression of brown stain nuclei for group A,B,C and D with negative expression for group E (40X).

Positive control Oral metformin I.P metformin 5-FU Negative control

Fig. 3 illustrates that metformin has no a clear effect on the body weight of the mice whether given orally as Group B or i.p. route as in Group C. figure (4) clearly demonstrates that the body weight for the mice in the above two groups followed the same pattern like in the positive control group (Group A) and negative control group (Group E) without any significant differences between them ($p > 0.05$).

Effect of metformin and 5-FU on fasting blood sugar

The results presented in table (2) revealed that mice treated with 5-FU twice weekly for 4 weeks shows significant elevation in the level of serum FBS when compare with the level in the other groups.

The level of FBS in mice treated with metformin for 4 weeks either orally like in group B or intraperitoneally in group C, are statistically did not differ from positive control (group A) and negative control group (group E).

The body weights of mice in the group treated with 5-FU drug (Group D) was markedly reduced ($p < 0.05$) compared to other groups particularly in the last 4 weeks, while those mice in Group E (negative control group), showed gradual increase in the body weight during the six weeks of the experiment.

DISCUSSION

Interest in chemoprevention for CRC has increased over the last decade, following evidence from epidemiological studies that have shown significantly lower rates of CRC in individuals reporting the regular consumption of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) [14]. They may be useful for those people who are at risk for familial adenomatous polyposis (FAP) and Hereditary non-polyposis colorectal cancer (HNPCC) which contributed about 25% of CRC cases [15]. Celecoxib has been approved by FDA as an adjunct therapy in familial adenomatous polyposis (FAP) [3]. However, recent studies have mentioned that serious cardiovascular side effects are associated with the using of selective COX-2 inhibitor [5]. That is why scientists are still searching for safe, effective and cheap chemopreventive agent for CRC. Many studies involving cancer outcomes among diabetic patients receiving metformin therapy have provided preliminary evidence that this compound favorably influences cancer outcomes [6,7]. Formation of ACF has been used by many researchers as an evidence of the early stage of colorectal carcinogenesis [16,17]. Besides that, Michael *et al.* (1996) was evaluated the efficacy of 41 potential chemopreventive agents in CRC using inhibition of AOM-induced ACF as measure of efficacy[18]. This study revealed that metformin can prevent the early stage of CRC by suppression the number of ACF induced by azoxymethane (AOM) in the colon and rectum of mice. In fact, the significant difference between control group and metformin treated groups is comparable with the results of Hosono *et al.* (2010). In this study the metformin was given also by i.p. route. The idea behind using this route came from Memmott *et al.* (2010) research, who observed that metformin can prevent tobacco carcinogen-induced lung tumor genesis in mice more significantly when administered intraperitoneally than when given orally for 16 weeks [8]. Because the plasma level of metformin was higher after injection than oral administration. Comparing to negative control group, i.p administration suppress the ACF more efficiently than oral administration. 5-FU also used in this research and we compared the efficacy of it with metformin in the suppression the ACF. Larger suppression in ACF numbers (1.5 + 0.48 ACF/mouse) has been observed in mice received 5-FU. Comparing this suppression number with metformin treated groups there is a significant difference between metformin- treated mice orally and those treated with 5-FU while there is no significant difference if we compared this result with those mice that administered metformin Intraperitoneally. This result indicates that the efficacy of metformin when given i.p. is close to the efficacy of 5-FU. To evaluate the antiproliferative effect of metformin in comparison with 5-FU, cell proliferation assay had been done. The anti-PCNA is useful for the analysis of cell proliferation in paraffinwax embedded sections, using the immunohistochemical technique [19]. This study revealed that metformin definitely has antiproliferative effect when compared with positive control group that induced by AOM. All treated groups either with metformin or 5-FU (group B, C and D) showed high significant reduction in PCNA LI when compared with positive control group. The results are consistent with study of Hosono *et al.* [9]. Also, in this research the antiproliferative effect of metformin has been compared with 5-FU. The study showed that there is no significant difference between the antiproliferative activity of 5-FU drug and metformin when administered intraperitoneally. Data collected from this study demonstrate that metfromin does not change FBS significantly when compared with positive control or negative control groups, even when given intraperitoneally. This finding is in accordance with other study that demonstrated no significant changes in FBS, Fasting C-peptide, fasting insulin and lipid profile in either metformin or placebo patients when metfromin give 500 or 850mg twice daily for 12 weeks [20]. 5-FU causes hyperglycemia. The FBS was significantly higher in Group D than that in positive control or negative control group. Feng *et al*, (2010), studied the effect of 5-FU on plasma glucose level in rat. They concluded that 5-FU can induce hyperglycemia in rats when given at a dose 20mg/kg daily for continuous 5 days[21].Metformin and 5-FU are drugs having gastrointestinal side effects like anorexia, nausea, vomiting, abdominal discomfort, diarrhea that is clearly observed in some animals during experiment. Administration of metformin at a dose 250mg/kg/day has no effect on body weight compared with positive control or negative control group (either administered orally or Intraperitoneally). While significant drop in the weight was observed in mice received 5-FU at a dose 15mg/kg/day (six timed

weekly) in comparison with positive control group. Researches have been done to explain the mechanism of action of metformin in prevention of cancer including colon cancer. Metformin may involve AMPK activation (AMP-activated protein kinas which serving as an energy sensor) by means of an LKB1-dependent mechanism [22]. Hosono *et al*., (2010), have been shown that metformin suppresses colonic epithelial proliferation (i.e. ACF) via the inhibition of the mTOR pathway through the activation of AMPK. While Zhou Xiao-zhi *et al*., (2010), revealed that, Metformin can inhibit the growth of SW-480 cells mainly by blocking the cell cycle at G0/G1, down-regulating the expression of cyclin D1 and decreasing telomerase activity [23].

CONCLUSION

Metformin has the ability to prevent the colorectal carcinogenesis by suppression the formation of ACF without causing any change in the level of FBS during the course of the treatment. Both metformin and 5-FU have significant antiproliferative activity against proliferation of colonic epithelium. Intraperitoneal administration of metformin has more therapeutic effect and more antiproliferative effect than oral administration so as its efficacy become close to that of 5-FU without significant difference between them.

ACKNOWLEDGEMENT

We would like to express our thanks to the laboratory staff in the department of pathology and forensic medicine at college of medicine, Al-Nahrain University for their technical assistance. Special gratitude is also expressed to chairman of animal house department of the Center of Quality Control and Drug Research, Baghdad Dr. Ishrak for her helps and supports. The authors would like to thank Dr Hayder B Sahib for his help in editing this paper.

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