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Is Olive Oil Consumption Suitable for Colorectal Cancer? *In Vivo* Preliminary Studies on Azoxymethane-Induced Colon Cancer in Rats

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Abstract

The incorporation of olive oil in the diet may have promoting or inhibitory effects on colorectal cancer (CRC). Objective: In this study, azoxymethane (AOM) was used to mimic CRC in rats and the effect of olive oil was correlated with the cancer progression in the colon of the rats. Design: Six weeks old Sprague-dawley male rats were randomized into 4 groups namely, naïve, indomethacin, saline and olive oil. Main outcome measures: This study was to investigate the effect of olive oil on preneoplastic cancer properties on the colonic mucosal surface for any tumors and the aberrant crypt foci (ACF). The induction AOM for the CRC by subcutaneous injection of 20 mg/kg.Rats were given 10 mg/kg b.w. of indomethacin dissolved in 0.9% saline by oral gavage for 4 weeks (28 days) as a positive control group.The negative control group was given 0.9% sodium chloride solution. Results: The experimental treatment compound, olive oil, was administered orally with a dosage of 7% daily food intake for 4 weeks (28 days). At week 6 (day 42), all animals were sacrificed by cervical dislocation and the colorectum was excised for histological examination.Histological sections were achieved using a microtome and histological sections were observed using a microscope. The mean body weights of the rats at 42 days are naïve – 238.5 ± 33.2, positive control – 251.5 ± 31.8, negative control – 231 ± 2.8 and treated group 262 ± 28.3. A total 3 ACF were found in the negative group compared to other groups. The crypts appeared regular with circular luminal openings and were arranged closely packed together in the naïve group. Crypts in the positive and treated group also had a similar appearance like naïve group.Conclusions: Olive oil inhibits the preneoplastic cancer properties ACF and maybe an incorporate into diet during CRC treatment or management.

Keywords: Preneoplastic, Olive oil, Aberrant crypt foci, Azoxymethane, Colorectal, Cancer.

Introduction

Colorectal cancer (CRC), also known as, colon cancer, rectal cancer or bowel cancer, is a heterogeneous disease that occurs in the inner lining of the colon and rectum.[1] Cancer remains to be a global cause of morbidity and mortality and the incidence continue to rise [2,3]According to many reports, CRC is the second most common cause of cancer in women and third in men.[1,3,4]The carcinogenesis is a multifaceted process that involves an abnormal growth of the mucosal surface termed as a polyp which develops at a slow rate into a premalignant lesion if left untreated.[1] Normal morphology of the colonic lining consists of crypts in which its bases contain regenerative

stem cells through a series of mechanisms, it will eventually extrude the epithelial cells and replace crypt cells every five days.[5] The presence of aberrant crypt foci (ACF) is remarked as a feature of possible CRC malignancy. ACF is defined as focal lesions formed specifically in the colonic mucosa that are composed of one to multiple crypts.[6] The first description of ACF was reported by Bird (1987) in which he performed a light microscopic examination of methylene blue-stained whole-mount preparations of AOM-treated mice colonic mucosa.[7] The carcinogens that specifically induce colon tumours in rats include aromatic amines, derivatives of cyacin such as 1,2-dimethylhydrazine (DMH) and its metabolite azoxymethane (AOM), alkylureas and heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).[6,8–11]

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen and sulindac are well reported to exhibit anti-neoplastic activity for colorectal tumours[12]epidemiological studies have demonstrated that continuous therapy with NSAIDs offers real promise of chemoprevention and adjunct therapy for colon cancer patients. Tumour growth is the result of complex regulation that determines the balance between cell proliferation and cell death. How NSAIDs affect this balance is important for understanding and improving treatment strategies and drug effectiveness. NSAIDs inhibit proliferation and impair the growth of colon cancer cell lines when tested in culture in vitro and many NSAIDs also prevent tumorigenesis and reduce tumour growth in animal models and in patients, but the relationship to inhibition of tumour cell proliferation is less convincing, principally due to gaps in the available data. High concentrations of NSAIDs are required in vitro to achieve cancer cell inhibition and growth retardation at varying time-points following treatment. However, the results from studies with colon cancer cell xenografts are promising and, together with better comparative data on anti-proliferative NSAID concentrations and doses (for in vitro and in vivo administration. It has demonstrated several actions at different phases of the Wnt pathway and effects on total β-catenin protein reduction, decreased nuclear localisation and decreased β-catenin/TCF binding resulting in suppressed cyclin expression.[13] A study performed and explored the significance of indomethacin in DMH-induced Sprague Dawley rats for a short term treatment of 3 weeks at doses 2 mg/kg and 1 mg/kg daily.[14] The experiment denotes satisfactory inhibition of ACF formation for the higher dose treated group while 1 mg/kg daily treatment did not produce significant statistical difference with the control.[13]

The cooking oils may contain numerous substances that can either protect against or promote for CRC development.[15] Vegetable oil is considered to possess more beneficial ingredients such as oleic acid than animal fat sources such as the trans fatty acid.[16]Olive oil is a rich source of biophenols and squalene such as oleuropein, flavonoids, phenolic acids, lignans, tyrosol and hydroxytyrosol.[17,18]Several anti-cancer investigations of *O. europeae*-extracts reported that erythrodiol, a triterpenoid of olives, exhibits anti-proliferative and apoptotic actions in HT-29 human adenocarcinoma cells. [19]The inhibitory effect olive oil observed in cancer cell lines has been linked with the blockade of the G2/M phase cell cycle through the inhibition of cyclic adenosine monophosphate (AMP) response element binding protein (CREB) phosphorylation resulting in a downstream reduction of COX-2 expression. Another study demonstrated the apoptotic activity of maslinic acid in HT29 human colon cancer cells via mitochondrial apoptotic pathway (Reyes-Zurita*et al.*, 2009).As mentioned by Chen & Huang (2009), CRC is the only cancer that can be prevented by selecting appropriate food and lifestyle.[20]

As the dietary intake of olive oil abundant with ω-9 MUFA had significantly inhibited the formation of ACF as well as decreased colonic arachidonate levels in AOM-induced

rat CRC model. [21,22] It was observed that AOM-induced colonic mucosa had significant increase in prostaglandin E_2 (PGE_2) concentration. However, olive oil treated rats had suppressed tumour growth mainly due to the regulation of COX-2 expression involving prostaglandin (PG) and caspase-3 synthesis that leads to apoptosis[23].

There are various studies on olive oil in relation to cancer, however in this study, we intend to observe the chemopreventive effect in the early stage of carcinogenesis and assess with an olive oil that is commercially available in which they can incorporate into their diet.This study involved the introduction of olive oil into AOM-induced rats to evaluate the action of olive oil on the quantity and size of polyps in colon. The outcome of this study is expected to provide nutritionists with a better comprehension on the effects of olive oil on colonic tumours, so a better and effective dietary pattern may be devised to eliminate the risk of nutritional deterioration in CRC patients.

Materials and Methods

Chemicals

The induction of CRC in rats was performed using carcinogen AOM purchased from Sigma Aldrich, St. Louis, MO. The drug indomethacin purchased from Promega, U.S.A. Olive oil which was produced by Borges Agricultural & Industrial Edible Oils in Tàrrega, Spain.

Animal experiment

The protocol of animal treatment was approved by the Animal Ethics Committee of Universiti Brunei Darussalam (UBD) and UBD Office of Safety, Health & Environment (OSHE). The research ethics for using animals were reviewed and approved by University Research Ethics Committee (UREC), Universiti Brunei Darussalam (Ref: UBD/OAVCR/UREC/Dec18-02). An five-weeks-old, male, Sprague Dawley rats which weighed between 100 to 150 grams (g), were kept under standard conditions of 27±2°C with 70-80% humidity and 12h light/12h darkness cycle. Furthermore, the animals were divided into 4 groups namely, naïve, indomethacin, saline and olive oil. In addition, each group consisted of 6 rats which were housed in one cage. The experiment protocol is summarised in Figure 1.Saline group was given 0.9% sodium chloride (NaCl) solution which represented the negative control group. The rodents were orally administered with 10 mg/kg b.w. of saline for 4 weeks (28 days). Indomethacin group acted as the positive control group and were given 10 mg/kg b.w. of indomethacin dissolved in 0.9% saline by oral gavage for 4 weeks (28 days).The experimental treatment olive oil group was administered orally with a dosage of 7% daily food intake for 4 weeks (28 days).

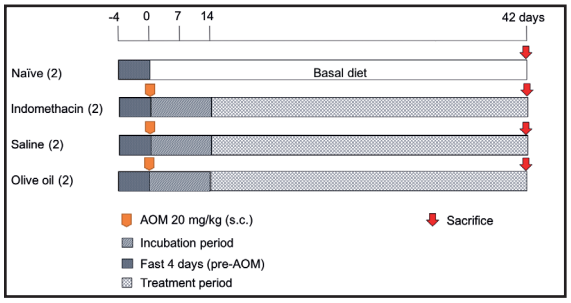

Figure 1 Experimental protocol for olive oil effect on CRC.

All rats were re-weighed before cancer induction on day 0 and each rat received 20 mg/kg body weight (b.w.) of AOM. All rats except naïve group received subcutaneous (s.c.) injection of AOM dissolved in physiological saline. The animals were allowed 2 weeks (14 days) of incubation period with access to food and water. On day 14, the animals began oral treatment which lasted for 4 weeks (28 days). Their body weights were measured weekly and macroscopically observed for the presence of rectal bleeding and diarrhoea. This group was neither given any carcinogen nor treatment compound. The naïve rats were only given basic food and water until they were sacrificed on day 42. Their body weights were recorded weekly and macro-

scopic observations were noted.

Macroscopic examination of ACF

After 24 hours of fixation, the colons were dipped in 0.2% methylene blue (Merck KGaA, Germany) in distilled water, then briefly washed with distilled water and placed on microscope slides with the mucosal surface uppermost. ACF was detected using light microscope (Olympus BX41, Japan) under 4x and 10x magnification.[6,24]distribution pattern along the colon and crypt multiplicity in 0.1% methylene-blue whole-mount preparations. ACF were distinguished from normal crypts by their larger size and elliptical shape. The incidence, distribution and morphology of colon tumors were recorded. The majority of ACF were present in the middle and distal colon of DMH-treated rats and their number increased with time. By the 4th week, 91.5% ACF were composed of one or two crypts and 8.5% had three or more crypts, while by the 30th week 46.9% ACF had three or more crypts. Thus, a progression of ACF consisting of multiple crypts was observed from the 4th to the 30th week. Nine well-differentiated adenocarcinomas were found in 10 rats by the 30th week. Seven tumors were located in the distal colon and two in the middle colon. No tumor was found in the proximal colon. The present data indicate that induction of ACF by DMH in the short-term (4 weeks The procedure involved counting the number of ACF in each colonic segment and evaluating the number of ACs in each ACF which was classified as either 1AC, 2ACs, 3ACs and ≥4ACs. After that, the tumours were prepared for haematoxylin and eosin staining for subsequent histopathological diagnosis.

Histological evaluation

Stained histological sections were observed using a microscope (Olympus BX41, Japan) at x10 and x20 magnifications. The cells were viewed on a computer using the software Olympus DP2-BSW and best representations of cells were photographed using a microscope digital camera (Olympus DP25, Japan). The histological classification was done according to a criteria described the cells may be categorised as normal, non-dysplastic ACF and dysplastic ACF.[5,25]The criteria for microscopic assessment was put into tabulated form below in Table 1.

Table 1. Criteria for microscopic classification.

Score	Criteria
0	Normal: straight test tube appearance, no serration, no branching.
1	Non-hyperplastic: enlarged crypts, lack significant abnormalities in epithelial cells, no mucin depletion.
2	Hyperplastic: larger and elongated crypts with side and apical branching, serrated lumen.
3	Dysplastic: nuclear stratification and enlargement, pleomorphism, hyperchromasia.

Statistical analysis

The data was statistically analysed using the GraphPad Prism software, Version 7. The numerical values for each group were expressed as mean±standard deviation (SD) and were subjected to unpaired *t*-test. The tumour multiplicity (number/rat) was expressed as mean±standard error of mean (SEM). For all cases, a value of $p<0.05$ was considered to be statistically significant.

Results and Discussion

General observations

The body weights were recorded each week for all groups. It was found that rats in all groups gradually gained weight throughout the experiment (Figure 2). However, inconsistency was found as the body weight was abruptly adjusted as seen in day 35 for naïve and positive,

and negative groups as well as day 42 for olive oil group. Rectal bleeding was not observed, but slight diarrhoea was noticed in the first week of treatment. Despite that, the rats consumed food as normal and were occasionally aggressive.

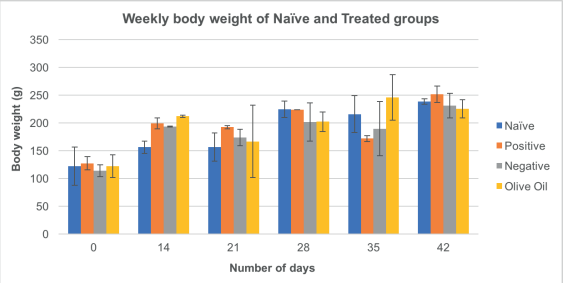

Figure 2. Effect of naïve and treated groups on body weight of Sprague Dawley rats.

Macroscopic evaluation

The mean length colon excised was 1.7 cm. The colon was divided into three portions in order to better manage for fixation and observing visible polyps. No visible neoplasm was detected in all groups. Figure 3 displays the normal appearance of rat colon with distinguishable features of proximal colon from distal regions.

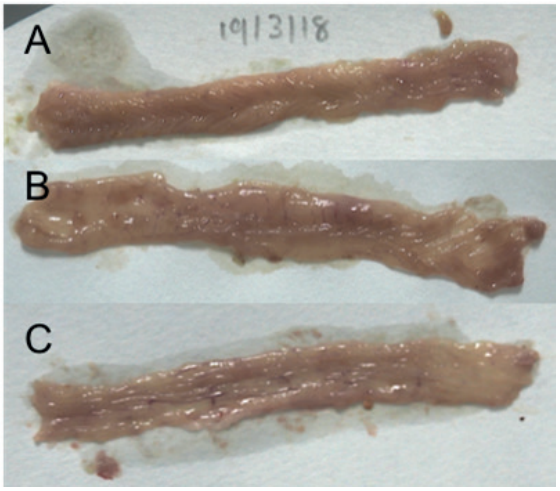

Figure 3. Macroscopic features of the colon. Whole colon was cut into 3 segments: (A) proximal, (B) middle and (C) distal. (A) Proximal colon can be easily distinguished due to visible foldings on mucosal surface. (B) Middle segment exhibits a transition from folded mucosa appearance of proximal to smooth mucosal surface in distal portion (C).

Effect of olive oil on ACF formation during initiation stages

Upon inspection under the microscope, it was found that negative and olive oil had an average of 0.5 and 4.5 of ACF respectively (Table 2) detected through the whole length of colon in each rat. Table 4 depicted that 0.5 ACFs in negative group contain 3ACs whereas olive oil had 4.0 of ACFs that contained 1AC and 0.5 ACFs with 2ACs. As the crypt multiplicity is higher in negative, formation and progression of ACF is more readily in this group than olive oil. As seen in figure 5, normal and positive groups exhibited normal configuration of crypts. Crypts in negative group displayed abnormal slit-like luminal opening, however this feature is not sufficient to consider as ACF. The best representation of an ACF is presented in negative (Figure 4) with obvious foci. Meanwhile, most slightly abnormal crypts found in olive oil group was not regarded as ACF.

Table 2. Effect of each treatment on AOM-induced colonic aberrant crypt foci (ACF) formation at week 6. Values are mean±SEM. No ACF were detected in naïve and positive groups.

Group	No. of ACF/ colon	Crypt multiplicity of ACF			
		1AC	2ACs	3ACs	≥4ACs
Naïve	0	-	-	-	-
Positive	0	-	-	-	-
Negative	0.5±0.5	0	0	0.5±0.5	0
Olive Oil	4.5±1.5	4.0±1.0	0.5±0.5	0	0

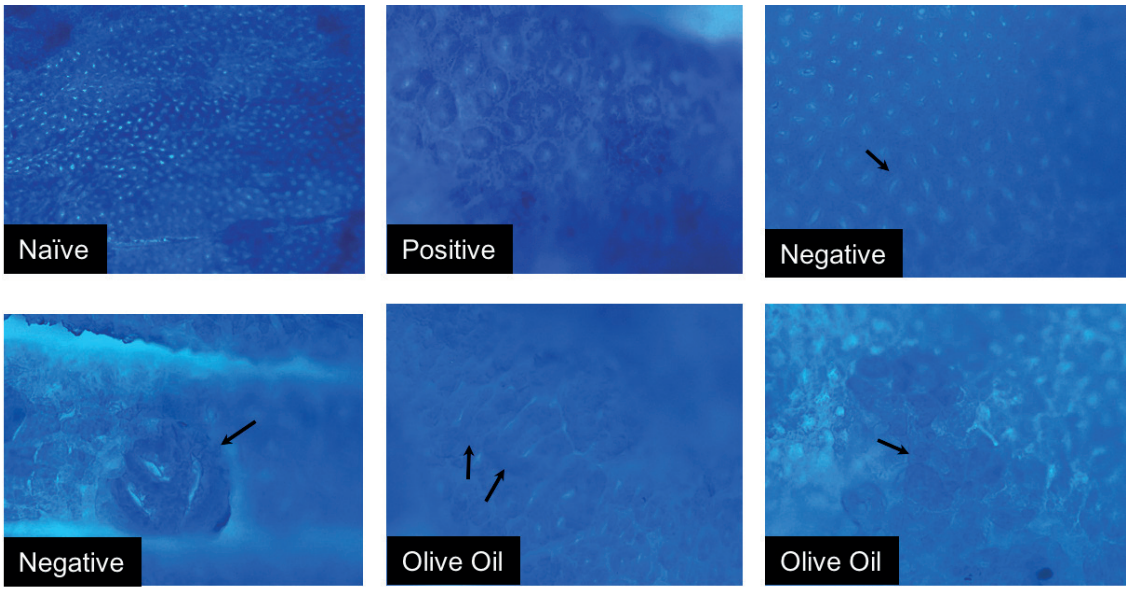

Figure4. A microscopic view of the whole mounts of methylene blue stained colonic showing presence of normal and aberrant crypts. No detection of ACF in naïve group and positive groups. Naïve at x4 magnification: exhibits crypts of similar size, round luminal opening and no significant abnormalities. Positive at x10 magnification: exhibits slight variation of crypt sizes with round luminal opening but no significant abnormalities. Negative at x10 magnification: most crypts had slit-like luminal opening (indicated with an arrow). Negative at x10 magnification: ACF detected was seen larger than normal and had thickened, darkened stain (indicated with an arrow). Olive oil at x10 magnification: exhibits increased dimensions with thicker and darker stain than normal (indicated with an arrow).

Non-hyperplastic cells were found in negative group only. The cells are indicated with arrows in Figure 5C and 5D. This finding supports the ACF that was macroscopically observed in Figure 4. Figures 5A and 5B illustrate the normal crypts as well as atypical crypts as seen in Figure 6 naïve, positive, negative slit-like lumen and olive oil. The microscopic scoring was based on numbering the categories normal (0), non-hyperplastic (1) and hyperplastic (2). The scoring values were averaged and presented in Figure 6. The naïve, positive and olive oil groups had normal scores in all colon segments while three segments in the negative group contributed to the mean 0.6 score. The negative group was found to be significantly different from naïve mean score. Whereas the olive oil aberrant crypt score is significantly lower than in negative control.cells. (D) Slight abnormal configuration of crypts with enlarged lumen (arrows).

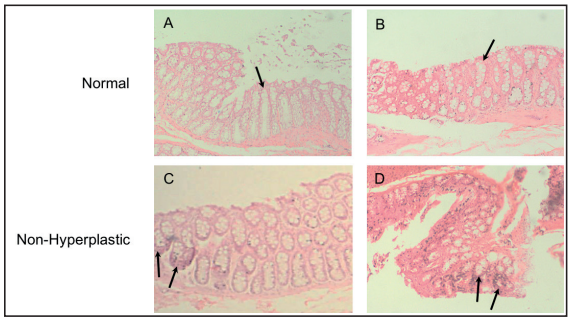

*Figure*5. A microscopic view of H&E staining detected for normal and non-hyperplastic histological category. (A, B) Normal mucosa showing characteristic straight test tube appearance, no serration and no branching indicated with an

arrow. (C) Crypts (arrows) are slightly enlarged but lack significant abnormalities in epithelial

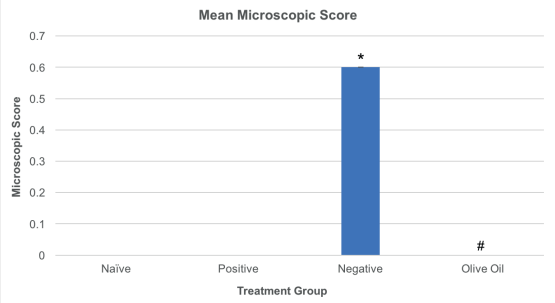

*Figure*6. Mean microscopic score of naïve and treated groups. Scoring is based on the criteria described by Alrawi et al. (2006) and Norlida & Phang (2010). Significant difference from naïve is denoted by *p<0.05 and from control is denoted by #p<0.05.

The use of animal models has become highly valuable for performing extensive investigative cancer studies due to its ability to demonstrate similar diseases in humans. The carcinogen AOM was used in this study due to its ability to promote cancer in the distal colon with high incidence in rats.There was no significance between the weekly weights gained in all groups. Several studies have attained similar insignificant weight gain of AOM-induced rats even in long-term experiments, however, there was no apparent explanation [26,27]6 weeks old, were injected once with one of five doses of azoxymethane. There was a dose response to the carcinogen as determined by weight gain and tumor induction. Rats given the three highest doses developed tumors of the gastrointestinal tract, auditory sebaceous glands, kidney, liver, and preputial gland, whereas rats receiving the lowest doses had tumors mainly of the intestine. Chronic liver lesions in high-dose rats were cirrhosis with megalocytosis, mild fibrosis, nodular hepatocellular hyperplasia, and hyperplasia of bile ductules.abstract":"BACKGROUND: Animal model studies have shown that the colon tumour promoting effect of dietary fat depends not only on the amount but on its fatty acid composition. With respect to this, the effect of n9 fatty acids, present in olive oil, on colon carcinogenesis has been scarcely investigated. AIMS: To assess the effect of an n9 fat diet on precancer events, carcinoma development, and changes in mucosal fatty acid composition and prostaglandin (PG. It is possible that the carcinogen itself did not affect the appetite of induced animals thereby carrying on with their typical food consumption. It is realised that weight is not an ultimate indicator of cancer symptom in animal models. However, the rats were observed to experience slight diarrhoea in the first week after induction which could be attributed to the metabolism of AOM irritating the mucosal lining as they are excreted in urine and faeces [20]. Even though the weights recorded in this experiment correlates with other studies, it was assumed that the values were inaccurate an unreliable on account of small population and fluctuating readings of the weighing balance. These issues can be overcome by increasing the population per group and using a weighing equipment with sensitive bearings.

The average length of colorectum documented is 1.8cm and the present study extracted an average length of 1.7cm [28]. This indicates that we are confident that we took into account appropriate parts for ACF observation. [11] Several studies have reported that AOM is able to stimulate ACF formation in colons as early as 5 weeks.[6,29] Since ACF detection is considered as biomarkers in short-term experiments, for that reason, the current study adopted an AOM-induced rat model that proceeded for 6 weeks. Even so, there were no visible neoplasms detected in all groups. ACF formation by AOM is well defined in distal part of colon and this corresponded to the locations of lesions we observed under the microscope. The indistinguishable inspection of polyps along the colonic mucosal surface could be argued on the basis of inadequate AOM dosage along with induction period, and misdetection of mucosal foldings with abnormal polyps. The severity of aberrant crypt formation may be improved by either in-

creasing the periodic injection to two injections a week for 2 weeks or once a week for the total experiment period 6 weeks [27,30]a constituent of olive oil, and a key intermediate in cholesterol synthesis may be regarded as partially responsible for the beneficial effects of olive oil, which include decreased mortality rates among populations with high olive oil consumption. Thus, in this study we have assessed the chemopreventive efficacy of squalene on azoxymethane (AOM.

The admittance of treatment compounds in day 14 after insufficient cancer induction could have restricted the growth of tumour. Additionally, the present experimental protocol could have created an ambiguity between the actions of test compounds affecting the carcinogenic potential of AOM from those that affected subsequent appearance and growth of lesions. This may be in which AOM was administered in two schedules of 15 mg/kg b.w. twice for 2 weeks and 30 mg/kg dose on day 7 only.[29] The test agent was given since the start of experiment at day 0 to 35 (5 weeks). Likewise, the study managed to detect few foci indistinguishable of the mechanisms mentioned previously. In order to confirm that ACF in the colon has been established before administrating test agent, a faction of rats should be sacrificed and inspected for polyp formation. This provides a basis for ACF count and multiplicity comparison in pre- and post-treatment administration[31]anti-oxidative, and anti-cancer properties but has poor bioavailability. Liquid crystals (LC.During the evaluation of this short-term investigation, it was observed that the positive control group did not develop any ACF and appeared normal as compared to negative control. Indomethacin is a potent NSAID known for its inhibitory effect on neoplastic growths. The research used a dosage of 3 mg/kg daily which was sufficient to prevent further growths as compared to the present study where 10 mg/kg b.w. daily was applied.[14,32]

It has been reported that ACF count and crypt multiplicity are predictive of colon tumour incidence. However, it was established that higher crypt multiplicity is indicative of malignancy and more aggressive tumour progression as opposed to high ACF counts. The negative control group exhibited a low number of ACF but with higher crypt multiplicity in comparison with olive oil group. The insignificant difference of ACF number and crypt multiplicity between olive oil and control groups suggests that dietary olive oil has a slight inhibitory effect on colon carcinogenesis. ACF with low crypt multiplicity is characterised as slower and less aggressive tumourigenesis. Since ACF was detected in negative control groups, microscopic observations displayed non-hyperplastic crypts in contrast with olive oil with morphologically normal crypts. Despite macroscopic observation of olive oil groups detected ACF growths, it was not reflected in microscopic examination. The morphogenesis of a colon tumour has been hypothesised to proceed in a top-down fashion whereby altered cells in the superficial mucosa proliferate laterally and downward to form new crypts adjacent to pre-existing normal crypts and eventually replace them [33]. It was assumed that technical skill errors in isolating abnormal tissue contributed to missed abnormal sections of tissues. The insufficient grade of abnormality in tissues may also cause the dismissal of aberrant categorisation. Tissue preparation could also contribute to the misdetection of crypts due to multi-layered tissue shaving leading to loss of aberrant compartment. This commercial 10% olive oil in the diet can significantly reduce the percentage of fragmented DNA, diminish ACF numbers and crypt multiplicity as well as induce apoptosis of tumour growths.[21,34]The present study utilised olive oil in the proportion of 7% of daily intake. This may account for the low inhibitory actions of olive oil on tumourigenesis in the study.

Low crypt multiplicity detected in the olive oil group showed promising significance of the compound in correcting DNA fragmentation that accounts for dysregulation of pathways

involved in cell proliferation and apoptosis. Although the present study did not manage to clarify the pathway olive oil predominantly affects, that may be due to high ω-9 MUFA as well as phenolic contents of olive oil might have a major influence in regulating Wnt pathways, subsequent COX-2 expression and arachidonate acid metabolism.[21,27,35]the effect of n9 fatty acids, present in olive oil, on colon carcinogenesis has been scarcely investigated. AIMS: To assess the effect of an n9 fat diet on precancer events, carcinoma development, and changes in mucosal fatty acid composition and prostaglandin (PG Furthermore, the present 6-week study has indicated that short-term experiments are inadequate to fully justify the questions of olive oil impact in early stages of cancer or even in high-risk individuals with predisposing mutations. Modulators of carcinogenesis such as COX-2, β-catenin, cyclin D1, Wnt 3, Wnt 5a and iNOSin the apoptotic activity and DNA fragmentation by western blotting should be investigated to recognise their roles in carcinogenesis.With these extensive findings, it would produce a highly reliable and credible outcome of the study to encourage nutritionists and the public to consume olive oil due to its potent beneficial properties during the CRC chemotherapy to avoid recurrence of cancer.

Conclusion

ACF formation is regarded as a precursor in CRC carcinogenesis of humans and rodents. Due to the ability to create a model that mirrors the disease in humans, the predictive value of ACF evaluation has become crucial in the context of anti-cancer drug evaluation. In this study illustrated that the dietary administration of olive oil in initiation stages of tumourigenesis has an inhibitive effect to some degree. The outcomes of the study emphasised the importance of conducting further investigations with olive oil, especially in long-term cancer models.

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Competing interests

The authors declare that they have no competing interests.

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