RESEARCH ARTICLE

Canola Oil Influence on Azoxymethane-induced Colon Carcinogenesis, Hypertriglyceridemia and Hyperglycemia in Kunming Mice

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Abstract

Azoxymethane (AOM) is a potent genotoxic carcinogen which specifically induces colon cancer. Hyperlipidemia and diabetes have several influences on colon cancer development, with genetic and environmental exposure aspects. Here, we investigated plasma lipid and glucose concentrations in Kunming mice randomized into four groups; control (no AOM or oil exposure), AOM control, AOM + pork oil, and AOM + canola oil. Aberrant crypt foci (ACF), plasma cholesterol, plasma triglyceride, plasma glucose and organ weight were examined 32 weeks after AOM injection. Results revealed that AOM exposure significantly increased ACF number, plasma triglyceride and glucose level. Further, male mice displayed a much higher plasma triglyceride level than female mice in the AOM control group. Dietary fat significantly inhibited AOM-induced hypertriglyceridemia, and canola oil had stronger inhibitory effect than pork oil. AOM-induced hyperglycemia had no sex-difference and was not significantly modified by dietary fat. However, AOM itself not change plasma cholesterol level. AOM significantly increased liver and spleen weight in male mice, but decreased kidney weight in female mice. On the other hand, mice testis weight decreased when fed canola oil. AOM could induce colorectal carcinogenesis, hypertriglyceridemia and hyperglycemia in Kunming mice at the same time, with subsequent studies required to investigate their genome association.

Keywords: Azoxymethane - colon cancer - hypertriglyceridemia - hyperglycemia - dietary fat

Asian Pac J Cancer Prev, **15 (6)**, 2477-2483

Introduction

Cancer is a widespread pubic health problem both in developed and developing countries, with environmental pollutants such as 2.5PM, or secondary hand nicotine smoke one of many influences on cancer development now affecting government policy (Park et al., 2013), including azoxymethane, which is an indirect mutagen and a specific colonic genotoxic carcinogen (Xiao et al., 1996). It is a genetic background-dependent colorectal cancer initiator and promoter (Anika et al., 2005). AOM-induced colorectal cancer animal model has been widely used in research to investigate the pathology and genetics of colorectal cancer (Hu et al., 2002; Raju 2008; Xiao et al., 2008).

In addition to environmental pollutants, obesity, insulin resistance and activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, are risk factors for colon cancer (Shimizu et al., 2009). In addition, diabetes and hypertriglyceridemia are important risk factors of colorectal cancer (Tabuchi et al., 2006; Kim et al., 2007; Ren et al., 2009; Vinikoor et al., 2009). High fat diet can induce carcinogen biotransformation enzymes, and also enhance colon tumour-genesis (Day et al., 2013; Diggs et al., 2013). Further, diabetes, hypertriglyceridemia and colon cancer are all genetically-dependent diseases (Pennacchio et al., 2003; Grady et al., 2008; Vaxillaire et al., 2009). These diseases are clinically interrelated to each other and can be modified through the diet. However, whether these diseases are genetically inter-related is not known. No animal model has been set up so far to induce and study all these diseases in the same animal.

To date a number of food ingredients have conferred protective effects against subcutaneous injection of AOM, and hence the investigation of the effect of commonly consumed oil, a major part of Chinese cooking, is important to research. The induction of colon cancer in mice, hamsters and rats including; Pomegranate (*Punica granatum*) peel extract (Waly et al., 2012), Moringa (*Moringa oleifera* L) pod extract (Budda et al., 2011), green tea polyphenols (Xiao et al., 2008), myrcitrin (i.e. flavonoid) (Asano et al., 2007), and resveratrol (Gurocak et al., 2013).

The formation of the premalignant lesion can be specifically inhibited by dietary agents such as chlorogenic acid (Matsunaga et al., 2002), black tea (Sengupta et

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al., 2002), the co-enzyme CoQ10 (Sakano et al., 2006), Neem (*Azadirachta indica*) (Arakaki et al., 2006), Cardamom (Sengupta et al., 2005), which may be related to cyclooxygenase-2 inhibition (Shen et al., 2004). Further, grapefruit juice, and *Zizyphus spina*-Christi, suppresses AOM induced aberrant crypt foci development (Guizani et al., 2013, Madrigal-Bujaidar et al., 2013).

Other food products are needed to examine their protective effect against air-borne, water and food carcinogens, particularly in P.R. China where green tea is a suggested protective dietary agent against 2.5μm induced obesity (Cichello et al., 2013) and thus possibly colon cancer. Green tea is a known dietary agent that confers fat malabsorption properties and this reduction of fat absorption i.e. saturated fat in pork lard, and inhibit abdominal fat pad accumulation (Bajerska et al., 2011) and thus the relative risk of colon cancer development (Bardou et al., 2013).

In the present study, we examined the effects of AOM on colon tumorigenesis, plasma triglyceride level, plasma cholesterol level, and plasma glucose level in Kunming mice to observe whether AOM can induce colon cancer, and change the levels of plasma lipids and plasma glucose in Kunming mice at the same time. Our research findings can provide insight into whether a genetic association exists among colon cancer, hyperlipidemia and diabetes. Moreover, the modification effects of two Chinese traditional cooking oils (canola oil and pork oil) on colon tumorigenesis, plasma triglyceride, plasma cholesterol, and plasma glucose level in AOM-treated mice were also assessed. Lastly, the observed effect of AOM in combination with the consumption of either pork lard, or canola oil was reviewed in reference to organ development.

Materials and Methods

Azoxymethane (AOM)

AOM was purchased from Sigma Chemical Co. (St Louis, MO, USA). It was dissolved in saline for injection.

Dietary oil

Pork oil and canola oil were used to investigate the regulatory effect of dietary fat type on AOM-induced colon cancer. Pork oil was refined from the pork leaf fat by the laboratory. Canola oil was a local retail product made in China and purchased from a supermarket. The two cooking oils are commonly consumed by Chinese people. Pork oil has 10% of polyunsaturated fatty acid, 48% monounsaturated fatty acid, and 42% saturated fatty acid. On the other hand, canola oil contains 36% of polyunsaturated fatty acid, 58% monounsaturated fatty acid and 6% saturated fatty acid. Pork oil was used as a representation of saturated cooking oil, whereas canola oil was used as the representation of unsaturated cooking oil used typically in China.

Preparation of rodent chow

Powdered mice basic feed was provided by the Experimental Animal Center, Kunming Medical University, Yunnan Province, China. The ratio of the oil addition in the high fat feed was 10%, namely 1kg of cooking oil and 9kg of powdered mice basic feed were completely mixed and then made into rodent chow. Rodent chow was freshly prepared every two weeks and stored in fridge for use.

Animal

Kunming mice are a local crossbreed experimental animal and were obtained from the Experimental Animal Center, Kunming Medical University, Yunnan Province, China. Prior to experimentation, the use of the mice was approved by the Animal Experimentation and Ethnics Committee, Yunnan. Mice were housed in cages and maintained in a temperature- and humidity-controlled animal facility with a 12-hr light-dark cycle. Mice in each group were fed the corresponding rodent chow and dH_2O. Mice were observed daily for clinical signs of illness, and body weight was recorded every two weeks.

Experimental procedure

80 Kunming mice (n=40 male, n=40 female) at 5 weeks of age were randomized into 4 groups (20 mice/group, 5mice/cage): Basic Control Group (BCG), AOM Control Group (ACG), AOM + Pork Oil Group (POG), and AOM + Canola Oil Group (COG). Mice in BCG and ACG were fed the same basic rodent chow, mice in POG were fed 10% pork oil rodent chow, and mice in COG were fed 10% canola oil rodent chow. Mice in ACG, POG and COG were given four weekly AOM i.*p*. injections (10mg/kg, on day 7, 14, 21 and 28), whereas mice in the BCG were given normal saline injection. Mice were killed 32 weeks after the last AOM injection. Prior to sacrificing, mice were fasted for 12 hours to collect fasting blood samples. Plasma was yielded from blood samples and used for the examination of plasma total cholesterol (TC), plasma triglyceride (TG) and plasma glucose (GLU). Heart, liver, kidney, spleen and testis were isolated and weighed. The organ coefficient (OCE) was calculated as the organ weight divided by the body weight and multiplied by 100.

Measurement of plasma total cholesterol, triglyceride and glucose level

Blood was collected into tubes treated with sodium heparin solution immediately following collection, and then centrifuged to isolate the plasma for the measurement of TC, TG and GLU according to the manufacturer's instructions contained in the assay kits. Kits were purchased from Marker Technique and Science Ltd, China. Glucose was measured by GOD-PAP assay, cholesterol was measured by CE-COD-POD (cholesterol enzyme - cholesterol oxidase – peroxidise) assay, and triglyceride was measured by GPO-POD (Glycerophosphate Oxidase – Peroxidase) assay.

Statistical analysis

Results were expressed as mean±standard error of the mean (SEM). All data were analysed using SPSS version 13 statistical package. TC, TG, GLU and OCE were analysed using two-way ANOVA with correction for multiple comparisons. Differences were considered significant at $p \leq 0.05$.

Results

Effects on plasma total cholesterol, triglyceride and glucose level

The plasma TC, TG and GLU levels of the four groups were examined at the end of the research after mice were stopped feeding for 12 hours (drinking water was provided *Ad lib*). The result of TC level was shown in Table 1.

The result in table 1 shows no significant difference in TC plasma concentration between BCG and ACG both in male and female mice ($p>0.05$). TC level of female mice in BCG and ACG was significantly lower than that of male mice ($p<0.05$). It indicated that AOM could not change plasma TC level, and male mice had a higher plasma TC level than female mice.

Compared with the result of BCG or ACG, TC level increased in male ($p>0.05$) and female ($p<0.05$) mice in COG and POG. Consumption of the two cooking oils increased plasma total cholesterol level in both male and female mice, and pork oil showed stronger TC promotion effect than canola oil. The result of TG level is shown in Table 2.

AOM significantly promoted plasma triglyceride level in both male and female mice, with the TG level of male mice significantly higher than that of the female mice in ACG ($p<0.05$).

The high TG level induced by AOM significantly dropped when mice were fed a high fat feed of either COG or LOG ($p<0.05$). High dietary fat significantly down-regulated the high plasma TG level induced by AOM, and canola oil showed much stronger effect than pork oil ($p<0.05$). The TG level in COG dropped to the level that had no significant difference with BCG in both male and female mice. On the other hand, the TG plasma concentration decreased significantly only in male mice in POG. The results indicated that dietary fat, especially unsaturated fatty acids could protect both male and female mice from AOM-induced hypertriglyceridemia. The result of plasma glucose level is shown in Table 3.

Interestingly, AOM also significantly increased plasma glucose level in ACG ($p<0.05$). The elevation of plasma glucose had no evident sex difference. Although the plasma glucose level was down-regulated in both COG and POG compared with that in ACG, no significant difference existed among COG, POG and ACG ($p>0.05$). Canola oil and pork oil had no significant influence on the AOM-induced hyperglycemia.

Table 1. Plasma Total Cholesterol Concentration (mmol/L)

Group	TC (mmol/L)		
	Male Mice	Female Mice	Total
BCG	2.16±0.60	1.01±0.66	1.68±0.84
ACG	2.11±0.39	0.89±0.29	1.50±0.71
COG	2.54±0.24	1.59±0.56*,**	1.98±0.56
POG	2.59±0.43	1.80±0.54*,**	2.04±0.56

*Compared with the BCG, $p\leq0.05$; **Compared with the ACG, $p\leq0.05$

Table 2. Plasma Triglyceride Concentration (mmol/L)

Group	TG (mmol/L)		
	Male Mice	Female Mice	Total
BCG	0.69±0.22	1.04±0.41	0.82±0.33
ACG	3.18±0.49*	1.90±0.35*,***	2.54±1.24*
COG	0.84±0.36**	0.90±0.25**	0.87±0.30**
POG	1.61±0.23*,**,***	1.68±0.40*,***	1.66±0.35*,**,***

*Compared with the BCG, $p\leq0.05$; **Compared with the ACG, $p\leq0.05$; ***Compared with the COG, $p\leq0.05$; ****Compared with the male mice in the same group, $p\leq0.05$

Table 3. Plasma Glucose Concentration (mmol/L)

Group	Plasma Glucose (mmol/L)		
	Male Mice	Female Mice	Total
BCG	3.54±0.96	3.82±0.68	3.70±0.79
ACG	7.48±1.85*	8.14±1.18*	7.81±1.55*
COG	6.74±2.10*	7.82±1.22*	7.31±1.73*
POG	6.51±1.74*	7.72±2.08*	7.37±2.00*

*Compared with the BCG, $p\leq0.05$

Table 4. Organ Co-efficients in Different Groups in Total

Group	Heart (%)	Liver (%)	Kidney (%)	Spleen (%)
BCG	0.49±0.08	3.72±0.46	1.49±0.22	0.25±0.09
ACG	0.45±0.07	4.28±0.62*	1.39±0.29	0.47±0.24*
COG	0.46±0.08	4.28±0.63*	1.34±0.31	0.46±0.23*
POG	0.48±0.12	3.83±0.81	1.43±0.44	0.38±0.14*

*Compared with the BCG, $p\leq0.05$

Table 5. Organ Coefficients of Male Mice in Different Groups

Group	Heart (%)	Liver (%)	Kidney (%)	Spleen (%)	Testis (%)
BCG	0.54±0.09	3.62±0.66	1.64±0.24	0.16±0.05	0.76±0.13
ACG	0.49±0.06	4.76±0.33*	1.64±0.11	0.42±0.18*	0.65±0.10
COG	0.52±0.07	4.76±0.48*	1.59±0.23	0.54±0.31*	0.59±0.07*
POG	0.55±0.11	4.09±1.12	1.85±0.21	0.43±0.17*	0.70±0.08

*Compared with the BCG, $p\leq0.05$

Table 6. Organ Coefficients of Female Mice in Different Groups

Group	Heart (%)	Liver (%)	Kidney (%)	Spleen (%)
BCG	0.46±0.05	3.79±0.25	1.38±0.14	0.30±0.06
ACG	0.42±0.07	3.80±0.43	1.14±0.14*	0.52±0.30
COG	0.41±0.04	3.83±0.36	1.11±0.16*	0.38±0.07
POG	0.43±0.11	3.44±0.61	1.10±0.25*	0.34±0.11

*Compared with the BCG, $p\leq0.05$

Effects on organ development

In this study, the weight of heart, liver, kidney, spleen and testis were examined to observe if AOM and the two cooking oils could affect the organ development. The organ coefficients were summarized in total in Table 4.

The results in Table 4 show that the liver and spleen weight in ACG were significantly higher than those in the BCG ($p<0.05$). In particular, the spleen mass was nearly two fold when compared between ACG and BCG. Canola oil did not change the effects of AOM on organ development, but pork oil decreased the weight gain of the liver and spleen caused by AOM although the spleen weight in POG was still significantly higher than that in BCG.

To assess whether AOM acted differently in organ development between male and female mice, the OCE in male and female mice was analysed separately in Table 5 and 6.

The results showed that the organ development

between male and female mice had striking difference when treated with AOM. The change of organ development in male mice was the same as analysed in Table 4. AOM significant decreased the weight of kidneys, but did not change the weight of liver and spleen in female mice. The kidney weight of female mice in ACG, COG and POG was significantly lower than that in BCG ($p<0.05$), but no difference existed between ACG, COG and LOG. Compared with ACG, a significant weight decrease in the testis was observed in COG in male mice ($p<0.05$).

Discussion

AOM is a specific colonic genotoxic carcinogen (AJCP reference). The susceptibility to AOM-induced colon carcinogenesis varies greatly between different mouse strains (Suzuki et al., 2006). In this study, only a few colon cancers were observed in the AOM-treated Kunming mice. However, AOM significantly increased the number of ACF (data not showed here). ACF is a putative pre-neoplastic lesion of colon cancer (Ranjana, 1995). The present results indicated that AOM could induce colon tumorigenesis in Kunming mouse although Kunming mouse were not very sensitive to AOM-induced colon carcinogenesis, only development of the ACF pre-cursor.

Hyperglycemia may be caused by lipid metabolism dysfunction (Jang et al., 2013, Ezhumalai et al., 2014). The hypertriglyceridemia observed in ACG was not the result of AOM-induced hyperglycemia, because the plasma glucose concentration in COG was similar to the ACG, however plasma triglyceride concentration in the COG was significantly lower than that in ACG. In this study there was both hyperglycaemia and hyperlipidemia in ACG. Both obesity and diabetes are considered to be important risk factors for the development of colon cancer. In male non-diabetic Long-Evans Tokushima Otsuka (LETO) rats and Otsuka Long-Evans Tokushima Fatty (OLETF) rats (diabetic rats), when whole-body X-irradiated (using 4 Gy), the incidence of small intestine adenocarcinoma in the LETO and OLETF rats were 0% and 30%, respectively. The sub-cutaneous injection of 15 mg/kg AOM once weekly for 3 weeks, promoted the development of zymbal gland tumors in LETO and OLETF rats were 0% and 67%, small intestine adenocarcinoma of 0% and 43%, cecum/colon adenocarcinoma were 46% and 79% ($P<0.05$/$P<0.01$), respectively. Moreover, both incidences of fatty hepatocytes in OLETR were 63%, with significantly higher serum triglyceride and free fatty acid levels when compared with the LETO rats (Hafez et al., 2011). Thus there is a clear link between hyperglycaemia, cecum/ colon cancer development and hyperlipidemia. Peroxisome proliferator-activated receptor (PPAR) agonists are effective in diabetes (Seok et al., 2013), and hyperlipidemia (Takahashi et al., 2013) and netoglitazone (Chang et al., 2007) has been shown in a colorectal cancer (CRC) model in A/J mice decreased mitoses and increased apoptotic cells in the CRC, with increased mRNA expression of tumor suppressor protein MUC2 in treated versus placebo mice (Imchen et al., 2013).

It has been shown in a study of male CRJ: CD-1 (ICR®) mice (outbred mice), that the subcutaneous injections of MSG (monosolidum glutamate) (2 mg/g body weight) four times daily (*quater die sumendus*; q.d.s) induces obesity and diabetes, which in combination with injections of AOM (15mg/kg bw) further increases the relative risk of colorectal cancer development. Moreover, mRNA expression of insulin-like growth factor-1 receptor (IGF-1R, $P<0.01$) was increased in the MSG-AOM mice (Hata et al., 2012). Diet induced obesity is often associated with hypercholesterolemia (Neyrinck et al., 2013). Interestingly, feeding a diet containing either 1ppm or 10ppm pitavastatin to AOM injected (s.c. 15mg/kg bw) C57BL/KsJ-db/db (db/db) obese mice reduced the number of colonic pre-malignant lesions even though obesity was still prevalent (i.e. increased serum adiponectin, and decreased leptin). Moreover, total cholesterol and pro-inflammatory cytokines (i.e. TNF-α), interleukin -6, IL-18 were decreased in the serum. Further, in the colonic mucosa there was a decrease in COX-2 (Yasuda et al., 2010), clearly showing a link between inflammation and colonic cancer, and obesity (Tuominen et al., 2013). It maybe probable that the canola oil diet conferred an anti-inflammatory effect, reducing serum adiponectin, and pro-inflammatory cytokines in colon mucosa, and thus prevented the formation of ACF in AOM injected mice fed canola oil.

High dietary fat intake is an important risk factor of hypertriglyceridemia. In this study, however, we found that the two different cooking oils conferred protection against the AOM-induced hypertriglyceridemia, with canola oil in particular displaying a much stronger protective effect than lard oil. Given that the AOM-induced hypertriglyceridemia resulted from the genotoxic effect of AOM, the results in the present study indicate that dietary fat, especially polyunsaturated could inhibit the genotoxic effect of AOM, such as hypertriglyceridemia and associated insulin resistance. In a study of A/J x C57BL6/J mice (obesity prone) versus wild type mice, showed the involvement of epidermal growth factor receptors (EGFR) in controlled azoxymethane tumorigenesis using a normal (5%) fat diet, versus high fat western diet (20%). On a high fat diet, mice displayed more weight gain and also insulin resistance with significantly increased tumor and cancer incidence, suggesting EGFR as a pivotal gene up-regulated for obesity and tumour growth (Dougherty et al., 2009).

Although diabetes is a genetic-related disease, such as the mutation of mitochondrion genes (Berdanier, 2007), it can also be induced by environmental influences i.e. diet. In this study, we did not differentiate if the AOM-induced hyperglycemia was IDDM or NIDDM, however, the results clearly highlight that the genotoxic carcinogen AOM could induce hyperglycemia in Kunming mice. Insulin resistance, in particular as shown in muscle-specific insulin receptor knockout mice, can increase susceptibility to colon carcinogenesis (Ealey et al., 2008), as per increased visceral adiposity, which is a symptom of insulin resistance (Ealey and Archer, 2009). Moreover, dietary sucrose, and thus hyperglycaemia are further indicated in the development of ACF as rats fed sucrose, oligofructose, and inulin, had higher number of ACF versus rats fed only oligofructose and inulin, thus consumption of less digestible carbohydrates,

caecal fermentation of carbohydrates, leads to decreases tumour incidence and multiplicity (Jacobsen et al., 2006). Interestingly, supplementation with glucose (10% drink) in Fischer 344 rats lead to AOM enhancement of colon carcinogenesis through HMGB1 induction (Ohmori et al., 2010), whereas the anti-diabetic medication metformin attenuates AOM induced colon cancer aberrant crypt foci via the mammalian target of rapamycin (mTOR) pathway through the activation of AMPK/ AMP-activated protein kinase (Hosono et al., 2010), thus effecting insulin synthesis and secretion. Thus, dietary hypoglycaemic agents may be useful in the complementary treatment of colon cancer and associated aberrant crypt foci. Some natural dietary compounds include *Nigella sativa* (Kapoor, 2009) which have been used in the treatment of colon cancer. It may be worthy to mentioned, that the hypoglycemic effect of canola oil, and even protein supplementation may decrease hepatic insulin extraction or increased C-peptide clearance, possibly explaining the reduced hyperglycaemia seen in this study using canola oil. Further dietary modifications such as moderate (20-25%) dietary energy restriction attenuates colon carcinogenesis in the Zucker obese rat model via reduction of colonic transforming growth factor-β and cyclooxygenase isoforms in Zucker obese (fa/fa) rats (Raju and Bird, 2003).

Moreover, the consumption of phytochemicals such as EGCG in green tea can reduce obesity in a high fat diet (Cichello et al., 2013) i.e. saturated fat, pork oil diet, possibly reducing the incidence of AOM induced colon cancer (as per observations by Xiao et al., 2008), which may be linked to polyphenol induced fat malabsorption, and thus reduction of abdominal obesity. In combination with other phytochemicals mentioned previously maybe combined with different dietary oils to observe either an inhibitor or proliferative effect of AOM induced colon cancer. Thus, these foods, and phytochemicals present a dietary treatment for AOM induced colon cancer possibly via anti-carcinoma, anti-inflammatory and anti-obesity effects. In summary, it appears that reduced obesity, via lower calorie intake, low fat diet and/or low sucrose/simple carbohydrate intake and the associated reduction in insulin resistance maybe beneficial to reduce the risk of colon cancer or pre-malignant lesions, and in part explaining the observation that a polyunsaturated fat such as canola oil protects against AOM induced hyperglycaemia and tumour formation.

The change in organ development caused by AOM injection displayed a sex-dependent difference. AOM had significant dose- and strain-dependent effects, but had no sex-dependent difference in colon carcinogenesis (Anika et al., 2005). The effect of AOM on organ development could not be attributed to AOM-induced colon carcinogenesis. The change in organ development also had no evident relation with plasma glucose, triglyceride or cholesterol concentrations. Testis weight significantly decreased in male mice when fed canola oil feed. Erucic acid and sinigrin are toxic substances in canola oil which can inhibit the growth of animal. Even though erucic acid is present at 2% it may have growth retardation effects, particularly the haematological system in piglets i.e. platelet size (Innis and Dyer, 1999).

Genome-wide association studies have provided insights into the genetics of complex disease and traits (Manolio et al., 2008; McCarthy et al., 2008). In the majority of instances, the functional link between a genetic association and the underlying pathophysiology remains obscure. The present study indicated that AOM-induced colon cancer, hypertriglyceridemia and hyperglycemia had a genetic association. Further studies are warranted to investigate the mechanisms underlying how AOM induces hypertriglyceridemia, hyperglycemia and promotion of colon cancer in Kunming mice, and further what the genome association is among these diseases. Further, the use of other dietary substances such as phytochemicals present in beverages, food or herbal species for complementary treatment of AOM induced colon cancer. The present study demonstrates a genotoxic-related animal model for the study of the above mentioned diseases, and moreover that polyunsaturated fat such as canola/ canola is protective against the formation of pre-malignant lesions and colon cancer.

Acknowledgements

All authors contributed equally to the study; Xiaoqiong He for experimentation planning and writing the first draft, Simon Angelo Cichello editing the 1st draft for English and scientific content, developed and wrote the second and third drafts as well as discussion section of the manuscript. Jiali Duan, Jin Zhou for their role in the experimentation phase of the study and input into the manuscript.

We would like to thank Professor Graeme P Young, Dr. Ying Hu and Ms. Jean Winter in the Center for Cancer Prevention and Control, Flinders University, Australia for their instruction and supervision of Dr. Xiaoqiong He's AOM research in Australia. Ms. Linbo Fang provided technical assistance in the animal husbandry. Dr. Bin Sun provided initial English revision of the first draft manuscript. This work was supported by Kunming Medical College.

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