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Research Article

Dose-Dependent Effect of Cyanocobalamin on the Prevention of Colon Cancer: An *In Vivo* Study

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Abstract

Azoxymethane (AOM) is an oxidizing agent that is commonly used to induce cancer in rat colon. Experimental studies strongly suggest an association between glutathione (GSH) depletion and colon cancer. GSH is an essential intracellular antioxidant, where its *de novo* synthesis requires cyanocobalamin, vitamin B12, yet its role on the prevention of colon cancer pathogenesis was not well studied. This study was undertaken to investigate the dose-dependent effect of cyanocobalamin supplementation in relation to GSH biosynthesis as well as its protective effect against AOM-induced oxidative stress in rat colon. Twenty-eight adult Sprague-Dawley rats were randomly divided into four groups (7 rats/group): Control group that fed standard diet with no AOM injection; and AOM-treated groups which received AOM injection and were fed one of three different diets supplemented with cyanocobalamin as follow: 25 µg, 125 µg, or 250 µg cyanocobalamin/kg of the standard diet. All rats were continuously fed with diet and watered *ad libitum* for 16 weeks, then they were sacrificed, and the colon tissues were examined microscopically for histological changes and homogenized for biochemical measurements of cellular oxidative stress markers (glutathione, and total antioxidant capacity). The results showed that AOM caused pathological changes in the colonic mucosal tissues and increased oxidative stress markers in rat colonic tissues. Cyanocobalamin, in a dose-dependent manner, has significantly ameliorated the AOM-mediated insults in rat colon. The results of this study provide *in vivo* evidence that cyanocobalamin supplementation protects against AOM-induced colonic cytotoxicity via abolishment of its colonic oxidative damage.

Keywords: *Azoxymethane; Colon Cancer; Cyanocobalamin; Oxidative Stress*

Introduction

The rate of colon cancer has increased dramatically as compared to other types of cancers, and this increase was attributed to oxidative stress that synergize with other etiological factors for the colon cancer pathogenesis worldwide [1]. Azoxymethane (AOM) is a common carcinogenic agent; specifically, it induces colon cancer in experimental animals, similar to the pathogenesis of human sporadic colon cancer [2]. Oxidative stress is a metabolic condition under which cellular antioxidant capacity is not counterbalancing the oxidative damage induced by various insults such as, free radicals and environmental toxins which is a direct cause for cellular damages (lipid peroxidation, protein inactivation and DNA breakdown), eventually leading to many chronic diseases such as cancer [3].

AOM induces oxidative stress in colonic cancer cells via a mechanism that is mediated by glutathione (GSH) depletion [4]. GSH is the major intracellular antioxidant and it undergoes oxidation to the oxidized form, GSSG, when scavenging oxidative stress insults [5]. In healthy cells and tissues, more than 90% of the total glutathione pool is in the reduced form (GSH), and less than 10% exists in the disulfide form (GSSG), and GSH depletion is considered an indication of oxidative stress [6]. AOM leads to the generation of reactive oxygen species and oxidative stress which lead to GSH depletion and inhibition of antioxidant enzymes with a subsequent cellular damage and carcinogenic pathogenesis [4-6].

Adequate dietary intake of cyanocobalamin is crucial for supporting several metabolic pathways, especially the methionine cycle (Figure 1) [7]. Under conditions of low cyanocobalamin, the methionine synthase (a key enzyme in the methionine cycle) becomes hypoactive and leading to an accumulation of its precursor, homocysteine, and eventually to the impairment of homocysteine dependent-biosynthesis to GSH.

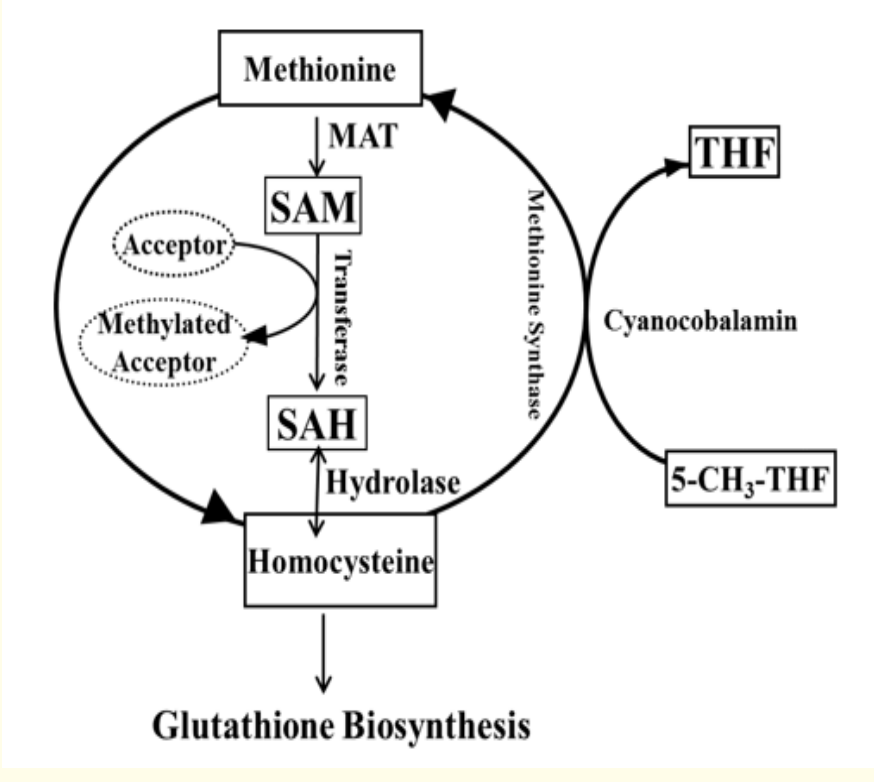

Figure 1: *Simplified schematic diagram of cyanocobalamin-dependent methionine cycle. Homocysteine is converted to methionine by methionine synthase enzyme, which utilizes cyanocobalamin as a cofactor and acquires a methyl group from 5-methyltetrahydrofolate (5-CH3-THF) which is converted to tetrahydrofolate (THF). Methionine is further converted to S-adenosylmethionine (SAM) through the activity of methionine adenosyl transferase (MAT). SAM is the major methyl donor for all methyltransferases, which adds methyl groups to various acceptor molecules and is then converted to S-adenosylhomocysteine (SAH), which is reversibly converted to homocysteine in a reaction catalyzed by hydrolase. Homocysteine is either remethylated back to methionine or transsulfurated to glutathione, the major cellular antioxidant.*

It has been documented that GSH depletion is associated with oxidative stress and increased cancer risk [8]. Several studies suggested that elevated tissue concentrations of reactive oxygen species are a pathological metabolite marker for oxidative stress and increased risk of colon cancer [9,10]. Although cyanocobalamin status is a major determinant of GSH, yet its etiologic relationship to oxidative stress in relation to colon cancer remains poorly understood. Accordingly, the present study was performed to investigate the potential protective effect of cyanocobalamin against AOM-induced carcinogenesis and oxidative stress in rat colon. Results of this study could enrich the ongoing chemoprevention research activities against colon malignancies.

Materials and Methods

Composition of the Experimental Diets: Ingredient values in the experimental diets were formulated according to the American Institute of Nutrition Rodent Diets, AIN-93M [11]. Modifications in cyanocobalamin content was made by adding pure cyanocobalamin (C3607, Sigma Chemical Co, St Louis, MI, USA) so that three different experimental diets were prepared with a net amount of 25, 125, or 250 µg cyanocobalamin/kg of the standard diet (Table 1).

Diet Ingredient (gm)	Standard Diet	25 µg Cyanocobalamin Supplemented Diet	125 µg Cyanocobalamin Supplemented Diet	250 µg Cyanocobalamin Supplemented Diet
Casein	140.000	140.00	140.00	140.00
L-Cysteine	1.800	1.800	1.800	1.800
Corn Starch	620.692	620.692	620.692	620.692
Sucrose	100.000	100.00	100.00	100.00
Fiber (α-cellulose)	50.000	50.00	50.00	50.00
Corn Oil	40.000	40.00	40.00	40.00
Mineral Mixture (AIN-93M-MX)	35.000	35.000	35.000	35.000
Vitamin Mixture (AIN-93-VX)	10.000	10.000§	10.000§§	10.000§§§
Choline Bitartrate	2.500	2.500	2.500	2.500
Tert-butylhydroquinone	0.008	0.008	0.008	0.008
Total	1000 gm	1000 gm	1000 gm	1000 gm

Table 1: *Composition of the experimental diets.*

Ingredient values in the experimental diets are given as grams (gm) of constituent per 1000 gm of the diet, which was formulated according to the American Institute of Nutrition Rodent Diets, AIN-93M [11]. §, §§, §§§*Modifications in cyanocobalamin contents were made by adding pure cyanocobalamin so that the net amount will be 25 µg, 125 µg, and 250 µg of cyanocobalamin/kg diet.*

Animal and Experimental Design: Twenty-eight Sprague-Dawley 6 - 8 weeks old male rats, with an average body weight of 200 ± 5g, were used in this experiment. The animals were maintained under standard laboratory conditions (temperature 22 ± 2°C, relative humidity 60%, and a 12 hr light: dark cycle) and allowed free access to diet and water *ad libitum*. The protocol used in this study was reviewed and approved by the Animal Ethics Committee (AEC) at the Sultan Qaboos University (SQU/AEC/2015-16/8). The Control group (n = 7 rats) was fed a standard diet with no AOM injection, meanwhile the AOM-treated groups (n = 21, 7 rats/group) were fed a cyanocobalamin supplemented diet (25 µg, 125 µg, or 250 µg cyanocobalamin/kg of the diet) and received AOM intra peritoneal injections (Figure 2).

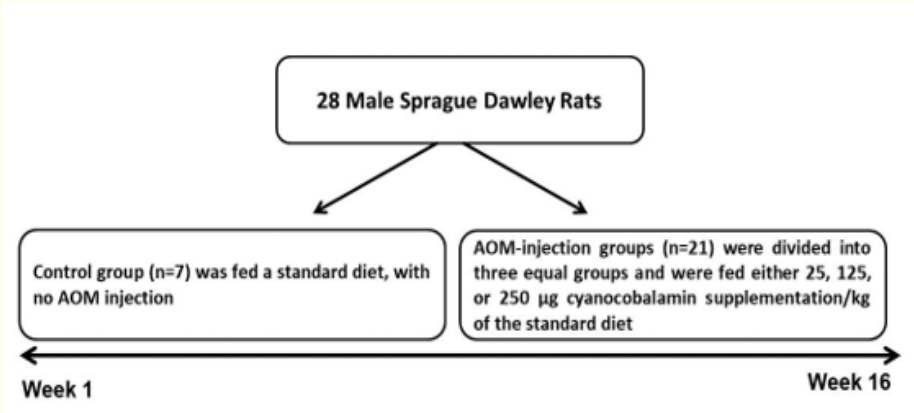

Figure 2: *Schematic presentation of the experimental design.*

Azoxymethane Injection: At week one of the experiment, the rats in the control group were given 1 ml intra peritoneal injection of 0.9% physiological saline once a week for 2 weeks and the rats in the azoxymethane (AOM)-injected group were given 2 intra peritoneal injections of AOM (Sigma Chemical Co., St. Louis, MI) dissolved in physiological saline once a week (15 mg/kg body weight) for 2 weeks, this dose was based on previous studies from our research group [2,4,5].

All animals were continuously fed *ad-libitum*, for the whole duration of the experiment. After 16 weeks, the animals were sacrificed by decapitation under diethyl ether anesthesia after an overnight fast and the colon tissues were removed and divided longitudinally into two equal parts: one part for microscopic examination for any morphological changes, and the second part was homogenized and use for biochemical analysis of the oxidative stress markers and protein measurement in colonic tissue homogenates.

Histopathology and Microscopic Examination: The colons were carefully removed from rats and were kept on a glass plate in ice jackets. The colons were then opened longitudinally, rinsed with ice-cold physiological saline, and were sectioned longitudinally into two halves of equal width and were spread out with flat mucosal side up. The mucosal layer from one half was removed by scraping and immediately homogenized. The other half was fixed flat in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ) between two filter papers for overnight and following tissue processing and paraffin embedding, sections were cut at 3 µm thickness and placed on glass slides; these were then stained with haematoxylin and eosin for colon architecture histology under light microscope examination (magnification x400).

Biochemical Analysis: The colonic mucosal layer scrapings of each rat (~50 mg) were immediately homogenized in 1 mL of 100 mM potassium phosphate buffer (pH 7.2) by a glass-Teflon homogenizer with an ice-cold jacket and centrifuged at 6,000g at 4°C for 20 minutes. The resulting supernatant was used for the measurements.

Analysis of protein content: Protein content of colon tissues was assayed by the method of Lowry., *et al*. using bovine serum albumin as standard and protein content was expressed as mg/ml of sample [12].

Glutathione (GSH) measurements: Aliquots of supernatant (100 µL) were transferred to fresh Eppendorf tubes and 2 µL of monochlorobimane (25 mmol/L) and 2 µL of glutathione-S-transferase reagent were added, as provided by a commercial kit (Biovision, Mountain View, CA, USA, Catalog # K251). After 30 minutes of incubation at 37oC, the samples and standards were read in a fluorescence plate reader at 380/460 nm. GSH content was determined by comparison with values from a standard curve using freshly prepared GSH and normalized to the protein content of the assayed colon mucosal tissue homogenates.

Total antioxidant capacity (TAC) measurements: lA colorimetric method using ELISA-Assay Kit (Biovision, Mountain View, CA, USA, Catalog # K247) was used to measure the TAC. The assay is based on the incubation of samples with 2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate (6)] diammonium salt (ABTS) with a peroxidase (metmyoglobin) and hydrogen peroxide to produce the radical cation ABTS+ which has a relatively stable blue-green color that is measured at 600 nm. Antioxidants present in the assayed colonal mucosal tissue homogenate samples inhibit the oxidation of ABTS to ABTS+ (cause suppression of the color production) to a degree that is proportional to their concentration. The capacity of the assayed samples antioxidants was compared with that of standard Trolox, a water-soluble tocopherol analogue, which is widely used as a traditional standard for TAC measurement assays, and the assay results are normalized to the protein content of the assayed colonal tissue homogenates.

Statistical Analysis

Data analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc. San Diego, CA). The results were expressed as means ± standard deviation (SD) of 7 independent observations from each group. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test, for means comparisons, and correlation between different variables was quantified by the correlation coefficient (r), P-value of less than 0.05 is considered significant.

Results and Discussion

All rats from all groups grew at a similar rate throughout the study, and no mortality occurred in any group. The mean weekly food intake for the all groups was measured during the experiment. All groups continued a consistent food intake throughout the experimental period and there were no statistical differences in food consumption between control and AOM-injected groups, $P > 0.05$. Daily food intake during the beginning (week 1) of the study was 12.1 ± 0.8 and 11.9 ± 0.4 g/day for control, and AOM-injected groups respectively. The same trend was observed during weeks 2 until the end of the experiment. On Week 16, there was an increase in food consumption but with no significant difference between control group (13.3 ± 0.6 g/day) and AOM-injected groups (13.6± 0.4 g/day).

Figure 3 showed the effect of AOM injections on the level of GSH, and it was observed that; the rats fed a 25 µg cyanocobalamin supplemented diet showed the lowest intracellular GSH content (8.75 ± 0.85 nmol/mg protein) of the colonic tissues homogenates as compared to the 125 µg and 205 µg cyanocobalamin supplemented diets (14.75 ± 1.2 and 20.18 ± 1.6 nmol/ mg protein respectively). The intracellular GSH level of AOM- injected groups was significantly lower than the control group rats fed on standard diet and with no AOM injections (20.18 ± 1.6 nmol/ mg protein), $F = 170.1$, R squared = 0.955, $P < 0.0001$. The correlation coefficient analysis revealed that among the AOM-injected rats, the intracellular GSH measurements tend to increase with the increase in the levels of cyanocobalamin supplementation. This positive correlation was statistically significant ($r = 0.95$, $P = 0.02$). Our data suggests that the AOM injections created an oxidizing milieu and induced the oxidative stress in colonic tissue by depleting the GSH. However, this depletion could be recovered by cyanocobalamin supplementation in a dose-dependent manner.

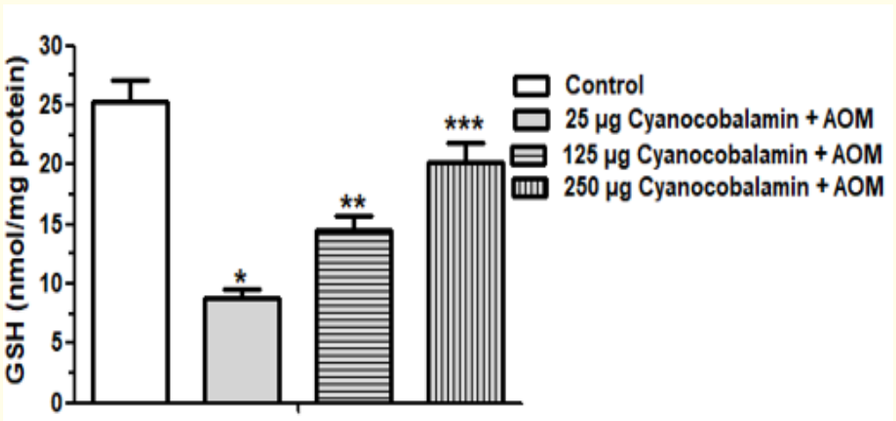

Figure 3: *Dose-dependent effect of cyanocobalamin supplementation in glutathione (GSH) measurement of colonic tissue homogenates. Data are expressed as mean ± SD. *, **, *** significantly lower than control group based on one-way analysis of variance (ANOVA) at P < 0.05.*

TAC is a cumulative action of all the antioxidants existing in the cells, therefore providing a unified parameter rather than the simple sum of countable antioxidants. As illustrated in figure 4, the mean TAC value of the control group was higher (148.56 ± 8.93 nmol/mg protein), as compared to the AOM-injected groups and supplemented with different doses of cyanocobalamin, F = 184.3, R squared = 0.958, P < 0.0001. In case of AOM-injected groups, the cyanocobalamin supplemented diets improved the TAC in a dose dependent manner (Figure 4). The highest TAC value was observed in 250 µg cyanocobalamin supplemented group (130.42 ± 6.42 nmol/mg protein), followed by 125 µg cyanocobalamin supplemented group (100.24 ± 4.21 nmol/mg protein). Meanwhile, the lowest TAC value was observed in 25 µg cyanocobalamin supplemented group (68.91 ± 6.85 nmol/mg protein). The pattern was statistically significant with a positive correlation (r = 0.95, P =0.02), indicating a protective effect of cyanocobalamin supplementation against the AOM-mediated impairment of TAC in the colonic tissue.

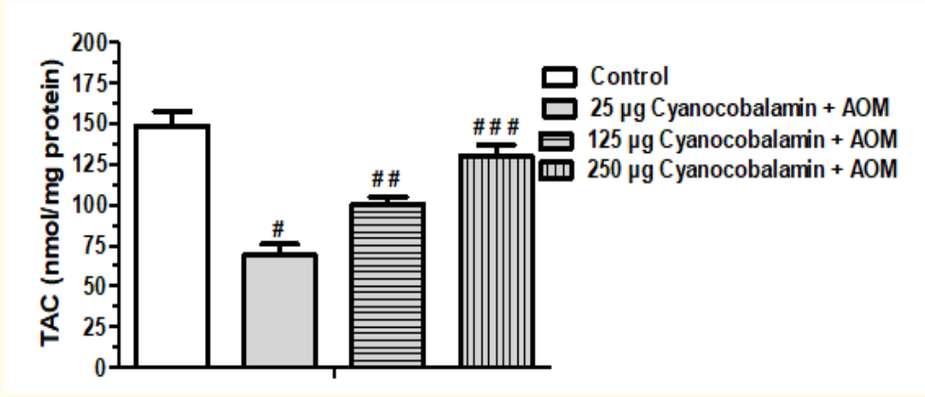

Figure 4: *Dose-dependent effect of cyanocobalamin supplementation in total antioxidant capacity (TAC) measurement of colonic tissue homogenates. Data are expressed as mean ± SD. #, ##, ### significantly lower than control group base on one-way analysis of variance (ANOVA) at P < 0.05.*

The examination of haematoxylin and eosin stained sections was reported in figure 5; for control samples the architecture of the mucosa was generally preserved in the control group (Figure 5A). Meanwhile for AOM-injected rats, the histologic examination of the colon reveals significant colonic mucosal abnormality like the increased muscle layer thickness of the muscularis mucosa, submucosa and muscularis propria. AOM-injected rats in the presence of 25 µg, 125 µg, or 250 µg cyanocobalamin supplementation showed dramatic improvement in the histologic appearance similar to the control rats (Figures 5B-5D).

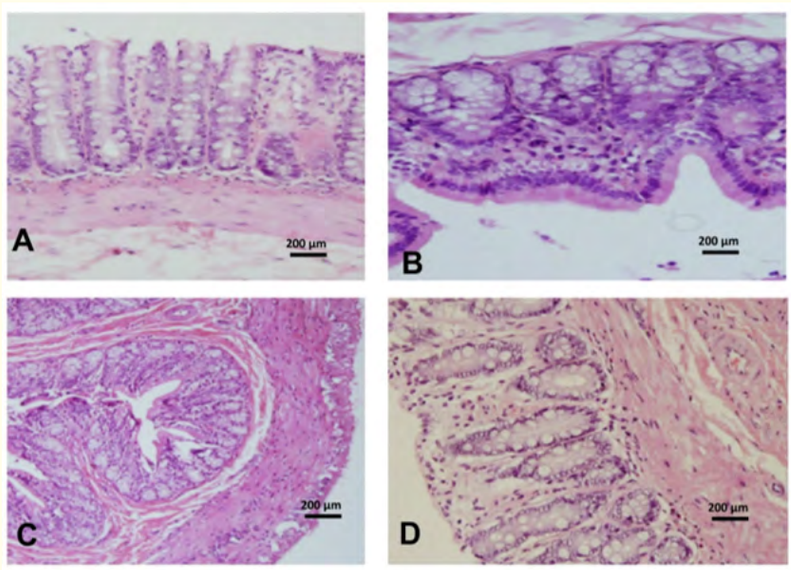

Figure 5: *Histopathological representation of colon tissues (haematoxylin and eosin stained sections, 400X). (A) Control rats with normal colon architecture. (B, C, and D) azoxymethane-injected rats in the presence of 25 µg, 125 µg, or 250 µg cyanocobalamin supplementation, respectively.*

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly produced in all aerobic organisms, mostly as a consequence of normal cellular aerobic respiration. Carcinogenic agents also trigger the production of abnormally high concentrations of unstable molecules, reactive oxygen and nitrogen molecules, in tissues and organs of various biological systems. The excessive production of ROS and/or RNS causes damage to DNA, proteins and lipids and can increase the risk of cancer. Antioxidants maintain redox homeostasis and prevent ROS-/RNS-induced damages that have been associated with cancer development. Oxidative stress is a condition under which glutathione and antioxidant enzymes (glutathione peroxidase, superoxide dismutase, and catalase), are not counterbalancing the ROS, and subsequently results in the impairment of cellular total antioxidant capacity with a damage in the cellular organelles and triggering the pathogenesis of cancer, including colon cancer.

In our study, the observed anti-oxidative properties of cyanocobalamin supplementation might be attributed to its modulating effect on glutathione biosynthesis, and this observation is based on the fact that the presence of cyanocobalamin with AOM treatment normalized the levels of the colonic intracellular glutathione to nearly normal and combats the observed AOM-mediated TAC impairment. Several investigators demonstrated that AOM induced reactive oxygen species in colonic cells and depleted intracellular concentrations of glutathione [13]. The increased proliferative activity seen in the examined colon samples for AOM-injured rats suggested a form of response to earlier or ongoing injury [14]. The epithelial cells lining of the crypts showed an increased turn over, which most likely represented a regenerative phenomenon due to AOM-mediated mitogenic effect [15]. A summary diagram for the cyanocobalamin supplementation protective effect against AOM-induced oxidative stress in rat colon is illustrated in figure 6.

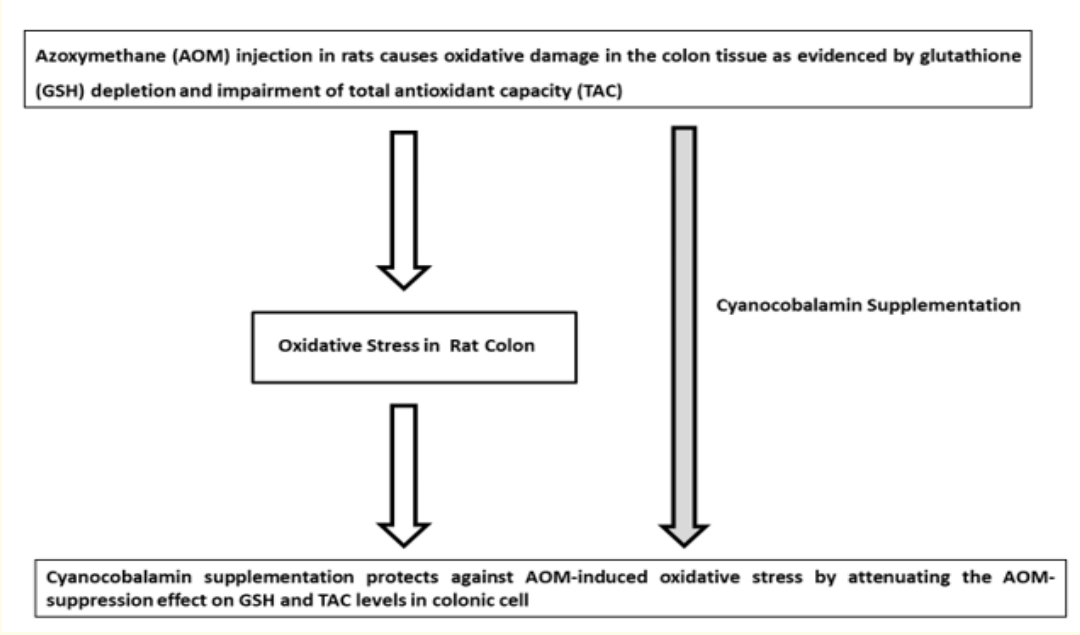

Figure 6: *A summary diagram for the cyanocobalamin supplementation protective effect against azoxymethane-induced oxidative stress in rat colon.*

Conclusion

Pathological changes in the colonic mucosal tissues and oxidative stress were associated with AOM injection. However, the concomitant treatments of AOM with cyanocobalamin supplementation have ameliorated the cytotoxic effects of AOM in a dose-dependent manner. Our study provides an *in vivo* evidence that cyanocobalamin mitigates the AOM-induced oxidative stress in rat colon, through combating glutathione depletion and its associated cellular insults.

Acknowledgment

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