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The melatonin action on stromal stem cells within pericryptal area in colon cancer model under constant light

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ABSTRACT

Constant light (LL) is associated with high incidence of colon cancer. MLT supplementation was related to the significant control of preneoplastic patterns. We sought to analyze preneoplastic patterns in colon tissue from animals exposed to LL environment (14 days; 300 lx), MLT-supplementation (10 mg/kg/day) and DMH-treatment (1,2 dimethylhydrazine; 125 mg/kg). Rodents were sacrificed and MLT serum levels were measured by radioimmunoassay. Our results indicated that LL induced ACF development ($p < 0.001$) with a great potential to increase the number of CD133(+) and CD68(+) cells ($p < 0.05$ and $p < 0.001$). LL also increased the proliferative process (PCNA-Li; $p < 0.001$) as well as decreased caspase-3 protein ($p < 0.001$), related to higher COX-2 protein expression ($p < 0.001$) within pericryptal colonic stroma (PCCS). However, MLT-supplementation controlled the development of dysplastic ACF ($p < 0.001$) diminishing preneoplastic patterns into PCCS as CD133 and CD68 ($p < 0.05$ and $p < 0.001$). These events were relative to decreased PCNA-Li index and higher expression of caspase-3 protein. Thus, MLT showed a great potential to control the preneoplastic patterns induced by LL.

1. Introduction

Colorectal cancer is one of the major causes of cancer death worldwide [1] and, constant light (LL) environment has been a promoter of colon tumors, mainly through changes in neuroendocrine colon system [2]. LL is associated with the downregulation of MLT release by neuroendocrine colonic cells promoting the colon cancer development [3,4]. As known, MLT exerts its oncostatic effects partially by its membrane and nuclear receptors [5], despite revealing a direct action against tumor cells [6,7].

A growing body of evidence supports that colon cancer is mainly driven by a small set of self-renewing cells, known as cancer stem cells (CSC) [8]. It has been well established that pericryptal colonic stroma (PCCS) covers stromal stem cells including the CSC cells, macrophages and other cell types [5]. Levi et al. [8] and Taketo et al. [9] have demonstrated that, PCCS provides a great variety of signaling molecules, growth factors and soluble mediators that contribute to the tumor growth, arising from a direct communication with tumor cells [10]. We have previously shown the main role of CSC cells in the colon carcinogenesis [11,12], besides revealing that tumor development and renewal reside in the CD133(+) cell sub-population, in accordance with xenotransplantation approach [13]. This minority of transformed stem cells supports the resistance against oncostatic agents in various solid tumors, including colon cancer [14,15], and may display a differentiated behavior against pro-apoptotic agents rather than those cells comprising the major tumor mass [16].

Based on the hypothesis that LL environment may act as promoter of initial colon cancer injuries as ACF and, that the MLT-supplementation is associated with prevention of colon cancer development, possibly through a cancer stem cell control, we investigated the action of MLT supplementation in animals exposed to LL as well as to the carcinogen 1,2 dimethylhydrazine (DMH). Moreover, we focused on the expression of stem cell, inflammatory, proliferative and, apoptotic cell markers at PCCS.

2. Materials and methods

2.1. Drug and carcinogen

MLT was purchased from Sigma Chemical Company (St. Louis, MO, USA). DMH (dimethylhydrazine) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

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2.2. Animals

Male wistar rats (150–160 g) were housed in a room at a mean constant temperature (22 ± 2 °C) with a 12-h light–dark cycle. They had free access to standard pellet chow and water. All experimental protocols were approved by the Animal Care and Use Committee (No. 142/2008) from the School of Medicine, University of São Paulo.

2.3. Experimental design

Animals were randomly divided into six groups with ten rats in each one: C/C was the control group; C/D received a single dose of 1,2 dimethylhydrazine (DMH; 125 mg/kg; i.p.); LL/C remained under LL environment (300 lx); LL/D remained under LL and received DMH-treatment; LL + MLT/C remained under LL and received daily MLT supplementation (10 mg/kg/day; i.p.); LL + MLT/D remained under LL and received DMH-treatment and MLT-supplementation. All animals were sacrificed at the 14th day post-DMH induction, and individual autopsies were performed. Blood was collected in sterile tubes and stored at −80 °C until analysis [17].

2.4. Radioimmunoassay for melatonin measurement

MLT serum levels were measured by radioimmunoassay using a commercial kit according to the manufacturer's protocol (Bio-Source Europe S.A., Nivelles, Belgium).

2.5. Histopathological analysis

We used standard procedures for identification and quantification of the aberrant crypt foci (ACF) in colon samples. This assay was performed by a pathologist as described elsewhere [18].

2.6. Immunohistochemistry

It was performed according to our previous description [19]. Briefly, we used primary antibodies provided by Novocastra®, Biocare Medical® and MBL®: NCL-PCNA (clone PC 10 at 1:100), NCL-COX-2 (clone 4H12 at 1:200), NCL-CPP32 (CASP-3) (clone JHM62 at 1:300), NCL-CD68-KP1 (clone KP1 at 1:200), CD133 (clone N/A 1:100) and Melatonin-Related Receptor (MLTr-Li; Polyclonal at 1:200). Positive reactions were detected in longitudinal sections as a brown precipitate in the nucleus for PCNA and, in cytoplasm and/or perinuclei for COX-2-Li (Labeling index), CASP-3-Li, CD68-Li, CD133-Li and, MLTr-Li. These patterns were scored into PCCS, the pericryptal-interval among whole crypts, each index was determined according to the total cell number related to positive cells. All analyses were performed by two independent observers, to avoid intraobserver bias.

2.7. Statistical analysis

Data were analyzed using the statistical program GraphPad Prism 5 (Graph Pad Software Inc., San Diego, California, USA). Analysis was performed by Kruskal–Wallis using Dunns posthoc test and Two-way ANOVA test with Bonferroni posthoc test. Probability of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. MLT serum levels and its receptor scores

Serum levels of melatonin (MLT) were not altered by LL regimen (Fig. 1A). MLT supplementation, on the other side, increased the amount of MLT found in serum samples ($p < 0.05$ and $p < 0001$). Besides of light regime or MLT supplementation, the addition of dimethyl hydrazine (DMH) significantly reduced MLT levels in comparison to the respective controls (Fig. 1A; $p < 0.05$, $p < 0.01$ and $p < 0.001$).

MLT receptor (MLTR-Li) was mainly detected in spindle-shaped cells of the PCCS spread through the crypt interval (Fig. 1B). MLTr-Li expression was markedly higher among MLT supplemented animals (Fig. 1C, $p < 0.001$). In animals treated with DMH, LL decreased the expression of MLT receptor compared to control animals kept under regular light conditions ($p < 0.05$).

3.2. MLT control upon pre-neoplastic injuries as ACF

In pathological analyses of colon tissue from DMH-treated animals, ACF clusters revealed enlarged nuclei, prominent nucleoli, nuclear polarity partly loss, higher mitosis numbers, and less globet cells. It was revealed that LL associated with carcinogen induced the highest number of hyperplastic and dysplastic ACF clusters ($p < 0.001$; Fig. 1D and E). At the beginning of malign injuries, as hyperplastic ACF, MLT did not reveal a preventive action to control their growth ($p < 0.01$; Fig. 1D). However, it was found that MLT supplementation significantly controlled the development of dysplastic ACF ($p < 0.05$ and $p < 0.001$; Fig. 1E).

3.3. MLT controlled the number of CD133 and CD68(+) cells in PCCS

CD133(+) cells were detected in higher numbers near reactive colonic areas into PCCS and in DMH-treated animals. Therefore, reactive areas were established according to malign features as stromal depolarization related to higher proliferative process taking their localization near to ACF clusters (Fig. 2A). Thus, LL environment, in association with carcinogen, induced the highest number of CD133(+) cells within PCCS ($p < 0,05$ and $p < 0001$). Furthermore, these cells were significantly decreased by MLT supplementation among DMH-treated animals and none significant difference among non-DMH treated groups was observed ($p < 0.05$; Fig. 2B).

Moreover, MLT-supplementation controlled the 2-fold increase of CD68(+) cells in those animals only exposed to the LL environment ($p < 0.05$). LL appears to increase the presence of CD68(+) cells among carcinogen-treated animals ($p < 0.05$). It was observed that MLT-supplementation controlled the 2-fold increase as initiated by LL exposure associated with carcinogen treatment ($p < 0.001$; Fig. 2C).

3.4. MLT induced a balance on proliferative and apoptosis patterns as well as on COX-2 expression

It was observed in animals which remained without carcinogen-treatment that MLT supplementation and LL exposure induced the 2-fold increase in the proliferative process into PCCS (PCNA-Li; $p < 0.01$ and $p < 0.001$). Nevertheless, MLT supplementation decreased the 2-fold increase in PCNA-Li cell numbers, as induced by LL exposure in animals under carcinogen treatment ($p < 0.05$), besides MLT groups were higher than the animals without LL exposure ($p < 0.001$; Fig. 2D). In addition, MLT induced the highest expression of CASP-3-Li protein within PCCS when compared to other groups exposed to the carcinogen ($p < 0.001$; Fig. 2E).

At last, MLT showed to control the 3-fold increase in the expression of COX-Li protein into PCCS among animals exposed to the carcinogen and LL, being them 2-fold lower than those under solely carcinogen treatment ($p < 0.001$; Fig. 2F).

4. Discussion

The findings revealed that LL environment for 14 days did not suppress MLT serum levels, despite being largely reduced by the

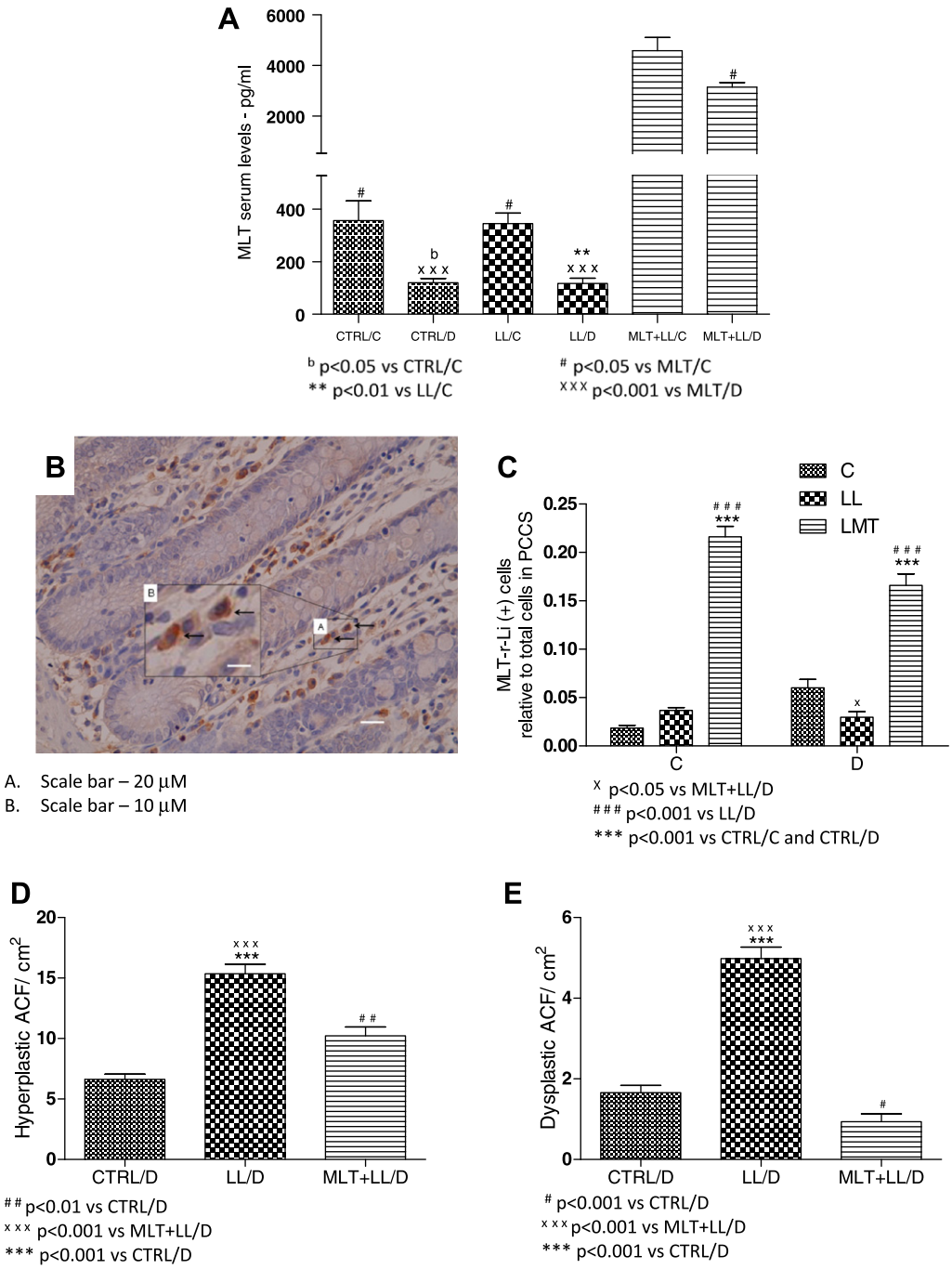

Fig. 1. (A) Melatonin (MLT) serum values according to radioimmunoassay in serum samples. *p* Values were determined by Kruskal–Wallis and Dunns posthoc test. (B) We found that MLT-receptor (MLT-r) was mainly expressed among stromal cells within pericryptal colonic stroma (PCCS) (A, ×400), and it was mainly labeled in the membrane and cytoplasm of spindle-shaped cells (B, ×1000). (C) MLT-receptor (MTL-r-Li; Labeling index) expression into PCCS in colon tissue. *p* Values were determined by Two-way ANOVA and Bonferroni posthoc test. (D) Hyperplastic aberrant crypt foci (ACF) index in colon tissue. (E) Dysplastic ACF in colon tissue. Number of ACF were demonstrated per cm^2 of colon mucosa in animals treated with 1,2 dimethylhydrazine. Bars and whiskers: Mean + SEM. *p* Values were determined by Kruskal–Wallis and Dunns posthoc test.

carcinogen treatment. Our findings lead to MLT supplementation as an inducer of the highest MLT levels even under LL exposure. This result has been previously reported when LL exposure was not able to suppress MLT levels despite its association with the disruption of the MLT rhythms [20]. Furthermore, several reports described that the carcinogen may induce a general decrease in MLT levels probably by a large amount of free radical molecules [21,22].

In the current study, we demonstrated that MLTr-Li expression appears mainly within PCCS, but the number of positive cells decreased after LL exposure when associated with carcinogen appliance. Surprisingly, MLT supplementation induced the highest levels of its both serum and receptor patterns. Emerging studies about LL environment have suggested that it blocks MLTr-Li expression possibly through molecular messengers of photoperiodic signals at transcriptional and translational levels [23]. Despite being a controversial point, previous reports have shown that oncostatic signals from MLT against LL effects are possibly supported by its receptor in the brain and colon tissue [24,25]. Thus, our collective findings about MLT supplementation are driven to the hypothesis that it may restored the blockade induced by LL

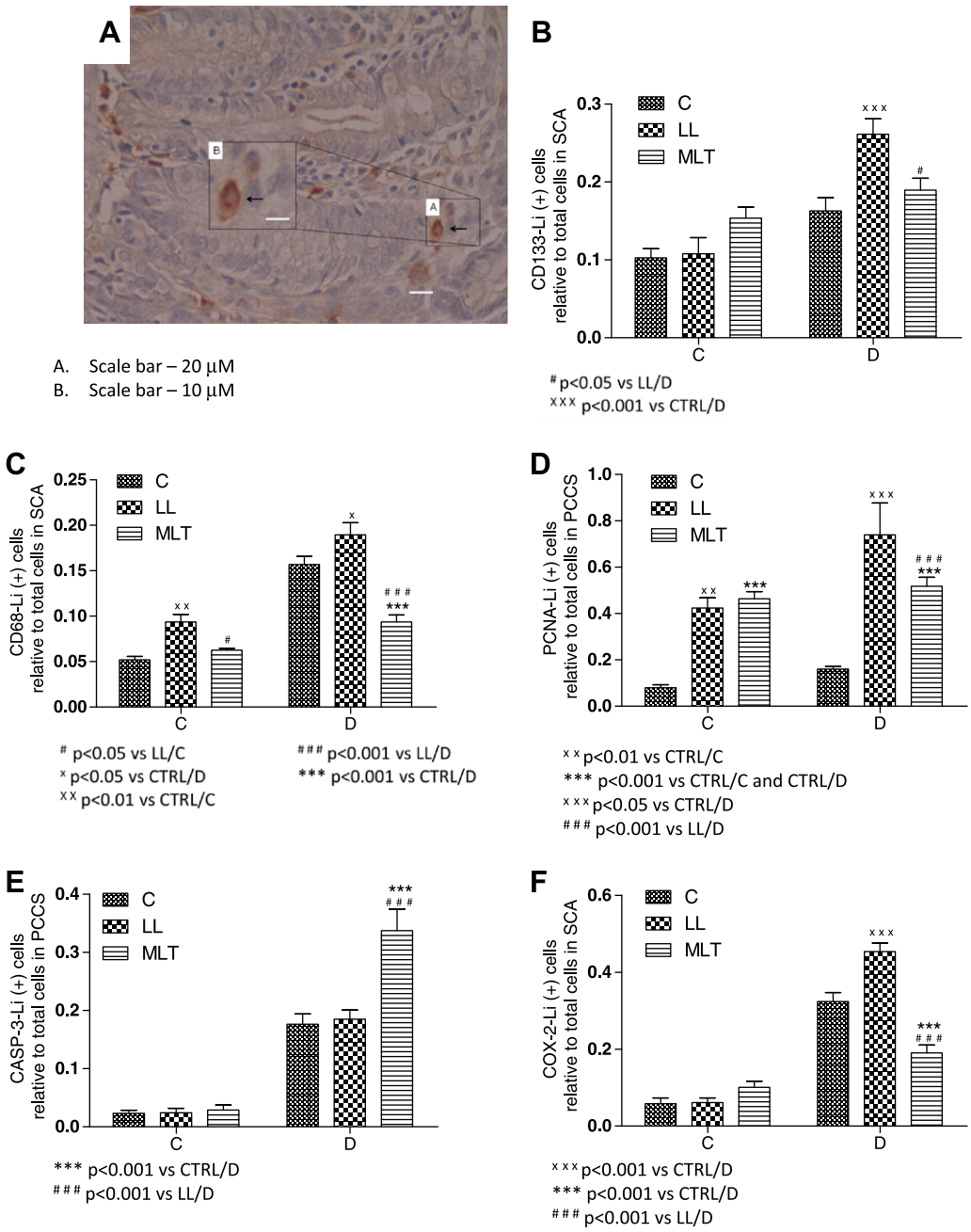

Fig. 2. (A) CD133(+) cells were detected mainly within pericryptal colonic area (PCCS), and near to reactive colon preneoplastic areas. It was observed a high tissue depolarization associated with high proliferative process and lower number of goblet cells (A, ×400). These cells were mainly labeled in cell membrane (B, ×1000). (B) Relative total number of CD133(+) cells into PCCS according to index of labeled cells by anti-CD133 antibody. (C) Relative total number of CD68(+) cells within PCCS according to index of labeled cells by anti-CD68 antibody. (D) Proliferative process (PCNA-Li) within PCCS established by index of labeled cells with anti-PCNA antibody. (E) Relative apoptosis process (CASP-3-Li) settled by index of labeled cells with anti-capase-3 antibody. (F) Inflammatory process related to cyclooxygenase-2 (COX-2-Li) expression into PCCS according to index of labeled cells by anti-COX-2 antibody. Bars and whiskers: Mean + SEM. *p* Values were determined by Two-way ANOVA and Bonferroni posthoc test.

environment upon MLT receptors within PCCS, then restoring the protective effects of MLT, partially related to the its receptor action.

The ability of LL to potentiate the preneoplastic changes as hyperplasic and dysplastic ACF, as shown in our results, were present into a short time of 14 days. MLT supplementation revealed a high potential to control mainly dysplastic injuries reducing their presence compared to control animals. Currently is known that LL potentiates colon tumorigenesis [4], moreover, a chronic MLT supplementation controls the development of colon tumors [26]. Therefore, MLT has modified the activity of cytochromes b5 and P450, a critical point to reverse the effects of carcinogen exposure [27]. In addition, MLT suppressed the formation of DNA-adducts due to its scavenger capacity associated with its nuclear accumulation and DNA stabilization [28].

The relationship between LL environment and CD133(+) cells growth is poorly reported besides being complex. Our present results suggest that LL exposure potentiated their growth after carcinogen treatment. It was revealed a pronounced MLT activity to control the enhancement of this subpopulation. O'Brien et al. [29], have shown that CD133(+) cells are inserted into carcinogenesis matter and may initiate and sustain the tumor growth, as well as the tumor relapse after chemotherapy [10]. At present many efforts have been applied in the development of anti-tumor agents that diminishes CSC cluster [13], mainly due to the high CSC cell

migration from PCCS towards the epithelium layer [30]. It has been shown that MLT plays a central role upon neural stem cell events, as proliferation and differentiation mainly by its receptor activity [31]. Besides, MLT has been reported as blocker of CD133(+) cells in tumor breast cell lines, event which directly supports our present results [32,33]. On the other hand, macrophage tumor cells have been controlled by MLT, probably controlling its proliferative process and possibly promoting intracellular reactive oxygen species (ROS) on a fast and transient way, not through an oxidative stress process [5,34] but by a possible re-localization of Bax/Bcl-2 at the mitochondria [35,36].

Our findings lead to LL potentiating a great imbalance between proliferative and apoptosis process within PCCS. It was found that MLT-supplementation controlled the growth of dividing cells as well as induced a higher expression of CASP-3 protein. Thus, we are suggesting an unknown capacity of MLT to control the development of preneoplastic cells since of early changes into PCCS. It has already been described that LL accelerates an uncontrolled gene expression in cell signaling, differentiation, proliferative, and apoptotic events possibly due to the deregulation of MLT cycle [37,38]. MLT has been suggested to induce apoptotic events in colon tumors [3]. Tanaka et al. [39], revealed that MLT inhibited the colon tumor developments possibly through a control of proliferative process associated with high apoptosis rates [40].

Our results corroborate that LL reinforces COX-2 expression, possibly controlled by MLT-treatment. Currently It is known that COX-2 protein potentiates the conversion of pro-carcinogens into carcinogens [41,42], being revealed that CD133(+) cells enhanced COX-2 expression in gliomas [43]. However, MLT has been shown to block the expression of COX-2 protein possibly by its receptor [44]. It has been previously shown, in colitis process, that MLT controls the overexpression of COX-2 protein, reducing colon damage and total number of macrophages within PCCS [45]. Moreover, MLT and its metabolites prevent COX-2 synthesis without affecting COX-1 protein levels [46].

Finally, we believe that MLT has a high potential to control malignant lesions in colon tissue possibly by an early action on PCCS changes, mainly upon the CD68(+) and CD133(+) cell clusters, possibly related to the high expression of MLT receptors. Despite the precise mechanism is unknown, we observed a relationship between the decrease of dysplastic injuries as ACF and, the lower presence of CD133(+) and CD68(+) cells. Possibly, it shows MLT as controller of proliferative patterns and inducer of the apoptosis process, a possible mechanism that controls the growth of CD133(+) and CD68(+) cells. COX-2 expression within PCCS was markedly reduced and may be associated with the balance of other PCCS outlines. Although MLT seems to control colon cancer development enhanced by LL, further studies should be accomplished.

Conflict of interest

The authors have no conflict of interest to disclosure.

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