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Fluoxetine induces preventive and complex effects against colon cancer development in epithelial and stromal areas in rats

Vinicius Kannen[a,*], Tassiana Marini[a], Aline Turatti[a], Milene C. Carvalho[b], Marcus L. Brandão[b], Valquiria A.P. Jabor[c], Pierina S. Bonato[c], Frederico R. Ferreira[d], Dalila L. Zanette[d], Wilson A. Silva Jr[d], Sérgio B. Garcia[a]

[a] Department of Pathology, Medical School of Ribeirão Preto, University of São Paulo, Ribeirão Preto 14049-900, Brazil
[b] Laboratory of Psychobiology, Faculty of Philosophy, Sciences and Letters, University of São Paulo, Ribeirão Preto, Brazil
[c] Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil
[d] National Institute of Science and Technology in Stem Cell and Cell Therapy, Center for Cell Therapy and Regional Blood Center, Ribeirão Preto, Brazil

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ABSTRACT

Fluoxetine (FLX) is a drug commonly used as antidepressant. However, its effects on tumorigenesis remain controversial. Aiming to evaluate the effects of FLX treatment on early malignant changes, we analyzed serotonin (5-HT) metabolism and recognition, aberrant crypt foci (ACF), proliferative process, microvessels, vascular endothelial growth factor (VEGF), and cyclooxygenase-2 (COX-2) expression in colon tissue. Male Wistar rats received a daily FLX-gavage (30 mg kg^{-1}) and, a single dose of 1,2 dimethylhydrazine (DMH; i.p., 125 mg kg^{-1}). After 6 weeks of FLX-treatment, our results revealed that FLX and nor-fluoxetine (N-FLX) are present in colon tissue, which was related to significant increase in serotonin (5-HT) levels ($P < 0.05$) possibly through a blockade in SERT mRNA (serotonin reuptake transporter; $P < 0.05$) resulting in lower 5-hydroxyindoleacetic acid (5-HIAA) levels ($P < 0.01$) and, 5-HT2C receptor mRNA expressions. FLX-treatment decreased dysplastic ACF development ($P < 0.01$) and proliferative process ($P < 0.001$) in epithelia. We observed a significant decrease in the development of malignant microvessels ($P < 0.05$), VEGF ($P < 0.001$), and COX-2 expression ($P < 0.01$). These findings suggest that FLX may have oncostatic effects on carcinogenic colon tissue, probably due to its modulatory activity on 5-HT metabolism and/or its ability to reduce colonic malignant events.

1. Introduction

Fluoxetine (FLX) is a selective serotonin reuptake inhibitors (SSRIs) with controversial effects on carcinogenesis, that was reported to be ineffective against aggressive T-cell lymphoma in nude athymic mice, despite the significant decrease of such tumors in BALB/c mice, in which it possibly acted on immune system to inhibit tumor growth (Frick et al., 2008). However, it has been shown to enhance apoptosis and control cell cycle in Burkitt lymphoma, in spite of not affecting the viability of non-tumor peripheral blood mononuclear cells (Serafeim et al., 2003). Meanwhile, FLX has been reported to promote metastasis formation in young transplanted melanoma mice (Kubera et al., 2009).

Once FLX is orally administered, it has a direct contact with the epithelia in the gastrointestinal tract (Arimochi and Morita, 2006), inducing an increase of serotonin (5-HT) levels by the blockade of serotonin reuptake transporter (SERT) (Bertrand et al., 2008). Moreover, high 5-HT levels have been implicated as promoters of cell proliferation and tumor growth in colon tissue (Tutton and Barkla, 1986). In accordance with the multiple colon tumor subpopulation theory (Barkla and Tutton, 1981; Garcia et al., 1999), tumor cell growth can be dependent or independent of colonic amine hormones, temporal switchover to hormone sensitivity and receptors activity (Barkla and Tutton, 1981).

We have previously shown that dysplastic aberrant crypt foci (ACF) induced by 1,2 dimethylhydrazine (DMH) is a well established method to study the colon cancer development in rodents and humans (Garcia et al., 2006; Wong et al., 2002) although, recent reports have implicated dysplastic ACF as a not predictable and characterized diagnosis method in human beings, restricting its applicability in clinical routine (Pinsky et al., 2010). Currently, this assay has been applied to detect inducer and/or modifiers factors in the early colorectal carcinogenesis (Garcia et al., 2006; Kannen et al., 2011), mainly due to its close relationship with the high cell turnover through an upward shift in the proliferation zone of the colonic crypts (Wong et al., 1999, 2002), leading to one of the first steps in the multistage colonic carcinogenesis (Garcia et al., 1999).

* Corresponding author. Tel.: +55 16 3602 3180; fax: +55 16 3602 1068.
E-mail addresses: kannen71@yahoo.com.br, viniciusfmrp@gmail.com, vinicius@daad-alumni.de (V. Kannen).

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Table 1
FLX, Nor-FLX, 5-HT, SERT mRNA, 5-HIAA, and 5-HT2Cr mRNA levels in colon tissues of Wistar rats treated or not with FLX and/or DMH.

Detection	CTRL/C	CTRL/D	FLX/C	FLX/D
FLX	–	–	35.24 ± 27.01	29.39 ± 19.21
Nor-FLX	–	–	89.09 ± 72.9	52.10 ± 16.22
5-HT	1.72 ± 1.51	0.77 ± 0.27	4.76 ± 2.94*	3.66 ± 1.75[b]
SERT mRNA	0.19 ± 0.09	0.09 ± 0.07	0.08 ± 0.03*	0.09 ± 0.04
5-HIAA	2.09 ± 0.97	0.79 ± 0.49	0.68 ± 0.19**	0.63 ± 0.04
5-HT2Cr mRNA	0.012 ± 0.01	0.017 ± 0.01	0.00029 ± 0.0003	0.00006 ± 0.00007[b]

FLX, fluoxetine; Nor-FLX, nor-fluoxetine; 5-HT, serotonin; SERT, serotonin reuptake transporter; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT2Cr, 5-HT-receptor. Mean ± SD, standard deviation.
[b] $P < 0.05$ *vs.* CTRL/D.
* $P < 0.05$ *vs.* CTRL/C.
** $P < 0.01$ *vs.* CTRL/C.

A growing body of evidence is increasingly supporting the idea that pericryptal colonic stroma (PCCS) activity is related to the high cryptal cell proliferation rates, since it expresses soluble factors that promote cancer-favorable transition and ACF development (Garcia et al., 1999; Kannen et al., 2011; Todaro et al., 2010). PCCS is located outside but adjacent to the basal lamina of cryptal epithelium in the lamina propria (Todaro et al., 2010; Valcz et al., 2011) and is associated with high vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) expression, contributing to malignant angiogenesis and colon cancer development (Liang et al., 2004; Park et al., 2011; Waldner et al., 2010).

The purpose of the present study was to verify the effects of FLX on 5-HT metabolism and recognition related to early malignant lesions in carcinogenic colon tissue. We focused on the hypothesis that FLX activity could endogenous upregulate 5-HT levels in a joint-activity to prevent dysplastic ACF development, which may be related to the proliferative process in colonic crypts. We also investigated this relationship in the modulation of malignant-microvessels development associated with VEGF and COX-2 expression within PCCS.

2. Materials and methods

2.1. Chemicals and solutions

FLX and nor-fluoxetine (N-FLX) were obtained from Research Biochemicals International (Natick, MA, USA). Moclobemide, used as internal standard (IS), was acquired from Roche Diagnostics (Mannheim, Germany). LC-grade methanol, acetonitrile, hexane, and isoamyl alcohol (P.A. grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Trifluoroacetic acid ammonium salt (98%) was purchased from Acros Organics (Morris Plains, NJ, USA). Sodium hydroxide was analytical-grade acquired from Spectrum Chemical MFG. Corp. (New Brunswick, NJ, USA). Purified water was obtained from a Milli-Q Plus system (Millipore, Milford, MA, USA). DMH was purchased from Sigma (St. Louis, MO, USA).

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. Experimental protocols were approved by the Animal Care and Use Committee (no. 150/2008) from the Medical School, University of São Paulo.

2.3. Experimental design

Animals were randomly allocated into four groups with six rats in each one. CTRL/C was the control group; CTRL/D received a single dose of DMH (125 mg kg^{-1}; intraperitoneal; i.p.) in the second week from the beginning of the experiment; FLX/C was given a daily FLX-gavage (30 mg kg^{-1}) for 6 weeks; FLX/D received daily FLX-gavage and a single dose of DMH. Rats were euthanized after 6 weeks from first FLX-gavage. Individual autopsies were subsequently performed, being the colon tissue piecemeal between frozen pieces (−80 °C) and fixed samples in formalin buffered solution by 24 h, as we previously described (Garcia et al., 2006; Kannen et al., 2011).

2.4. Detection of 5-HT and 5-HIAA by HPLC

As we previously described (Moreira et al., 2007), 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were quantified in frozen colon samples. They were quantified by comparing the peak areas to standard curves by the computer program Class-LC 10A (Shimadzu, Japan), being the concentrations expressed in ng mg^{-1} of colon tissue.

2.5. Drugs analysis in colon samples by LC–MS–MS

FLX and N-FLX were isolated from colon tissue samples (30 mg) according to our own method adapted (Borges et al., 2009). A Quattro LC triple quadrupole mass spectrometer (Micromass, Manchester, UK) was interfaced via an electrospray ionization (Z-ESI) probe with a Shimadzu (Kyoto, Japan) liquid chromatography, equipped with a LC-AT VP solvent pump unit. FLX, N-FLX, and IS were separated on LiChrospher® 100 PR-8, 5 μm, 125 mm × 4 mm column (Merck, Darmstadt, Germany). A C8 guard column (4 mm × 4 mm i.d., Merck) was used. Samples were separated under isocratic conditions using a mobile phase consisted of acetonitrile:0.1% trifluoroacetic ammonium acetate aqueous solution (60:40, v/v), at a flow rate of 1.3 mL min^{-1}. Quantification was performed by multiple reaction monitoring (MRM) of the precursor ions and their corresponding product ions. The precursor-to-product ion transitions were monitored at m/z 310 > 44 for FLX, m/z 296 > 134 for N-FLX, and m/z 269 > 182 for IS. A MassLynx data sampling and processing system (Micromass) version 4.1 was used.

2.5.1. Calibration solutions

Stock solutions of FLX and N-FLX containing 200 μg mL^{-1} were prepared in methanol. IS solution was prepared in methanol at 0.10 μg mL^{-1}. Calibration curves were obtained by analyzing spiked colon samples in duplicate over the concentration range of 6–500 ng of the drug per mg of colon.

2.6. Real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from frozen colon tissue samples (30 mg) using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. In the quantitative real-time PCR (qPCR), data were continuously collected and analyzed using ABI-7500 SDS software package. Total RNA input was normalized based on C_t values for GAPD housekeeping gene, as a reference standard. GAPD assay ID was 4352338E (Applied Biosystems). DNASTAR software (version 3.0) was used to design the primers sequence to amplify 253 and 197 bp of 5-HT2C (NM_012765) and SERT (NM_013034.3), that were amplified respectively using SiberGreen reagent (Applied Biosystems, Foster City, CA, USA). All reactions were duplicated, according to the standard 7500 software PCR program. The fold change was calculated using $2^{-\Delta C_t}$ method.

2.7. Histopathological analysis in colonic epithelia

The standard procedure was applied to identify and quantify the dysplastic ACF-I (index) in epithelia, and microvessels in PCCS. They were both performed by a pathologist as described elsewhere (Kannen et al., 2011; Skinner et al., 1995).

2.8. Immunohistochemistry (IHC)

As we previously described (Kannen et al., 2011), primary antibodies were provided by Novocastra®: NCL-SEROTp (1:100), NCL–PCNA (clone PC 10 at 1:100), SCB–VEGF (clone A-20 at 1:100), and NCL–COX-2 (clone 4H12 at 1:200). Positive reactions were detected in longitudinal sections as a brown precipitate in the nucleus for proliferative cellular nuclear antigen (PCNA) and in cytoplasm and/or perinuclei for SEROT (5-HT), VEGF-Li, and COX-2-Li. Cryptal proliferative cell index (PCNA-Li, labelling index) were expressed in each sample according to total cell number related to positive cells. To determine VEGF-Li and COX-2-Li scores in PCCS, the same criteria were applied. Staining procedure with anti-SEROT antibody was carried out to clarify its location in colon tissue. Analyses were performed by two independent observers, to avoid intraobserver bias.

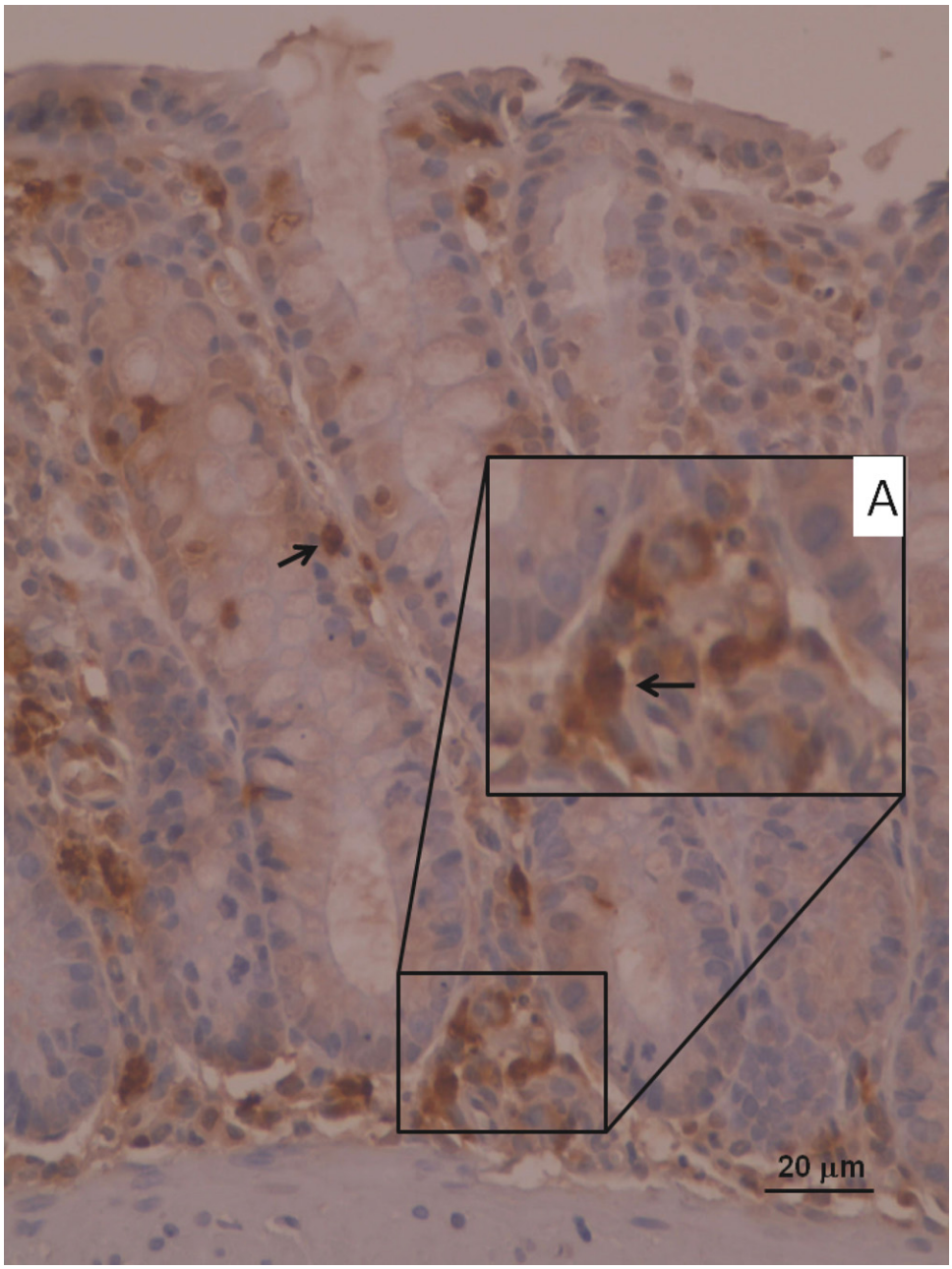

Fig. 1. Anti-SEROT (5-HT) antibody was applied at colon tissue sections, as a broad spectrum antibody to serotonergic activity. As indicated by the arrow, 5-HT activity was mainly detected among stroma cells within pericryptal colonic stroma (PCCS) and, mainly near to the crypt bottom, despite this, positive cells were detected in less extension in crypts (400×; scale bars 20 μm). (A) SEROT (+) cell is indicated by the arrow (high magnification).

2.9. Statistical analysis

Data were analyzed using the statistical program GraphPad Prism 5 (Graph Pad Software Inc., San Diego, CA, USA). Data were analyzed by two-way ANOVA test with Bonferroni post hoc test. However, for ACF and drug concentrations analysis, an Unpaired *t* test was applied. Probability of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. FLX and N-FLX modulate 5-HT and 5-HIAA levels through serotonin enteric transporter (SERT) in colon tissue in vivo

As shown in Table 1, FLX and Nor-FLX levels in colon tissue of rats given FLX by 42 days did not reveal any difference between DMH or non-DMH treated rats. As expected, FLX treatment significantly increased 5-HT levels at samples of colon tissue ($P < 0.05$) and significantly reduced SERT mRNA expression and 5-HIAA levels at non-DMH treated group ($P < 0.05$ and $P < 0.01$). DMH treated rats that received FLX revealed a strong downregulation of 5-HT2C receptors mRNA expression ($P < 0.05$). Anti-5-HT antibody shows, which serotonergic activity is mainly occurring in stroma cells within PCCS (Fig. 1). Moreover, DMH-treatment alone reduced SERT mRNA and 5-HIAA levels in colon tissue to the same levels detected in FLX-treated groups.

3.2. FLX controls over dysplatic ACF and microvessels development

FLX has been shown to be an oncostatic agent (Stepulak et al., 2008; Tutton and Barkla, 1982). However, its potential against the

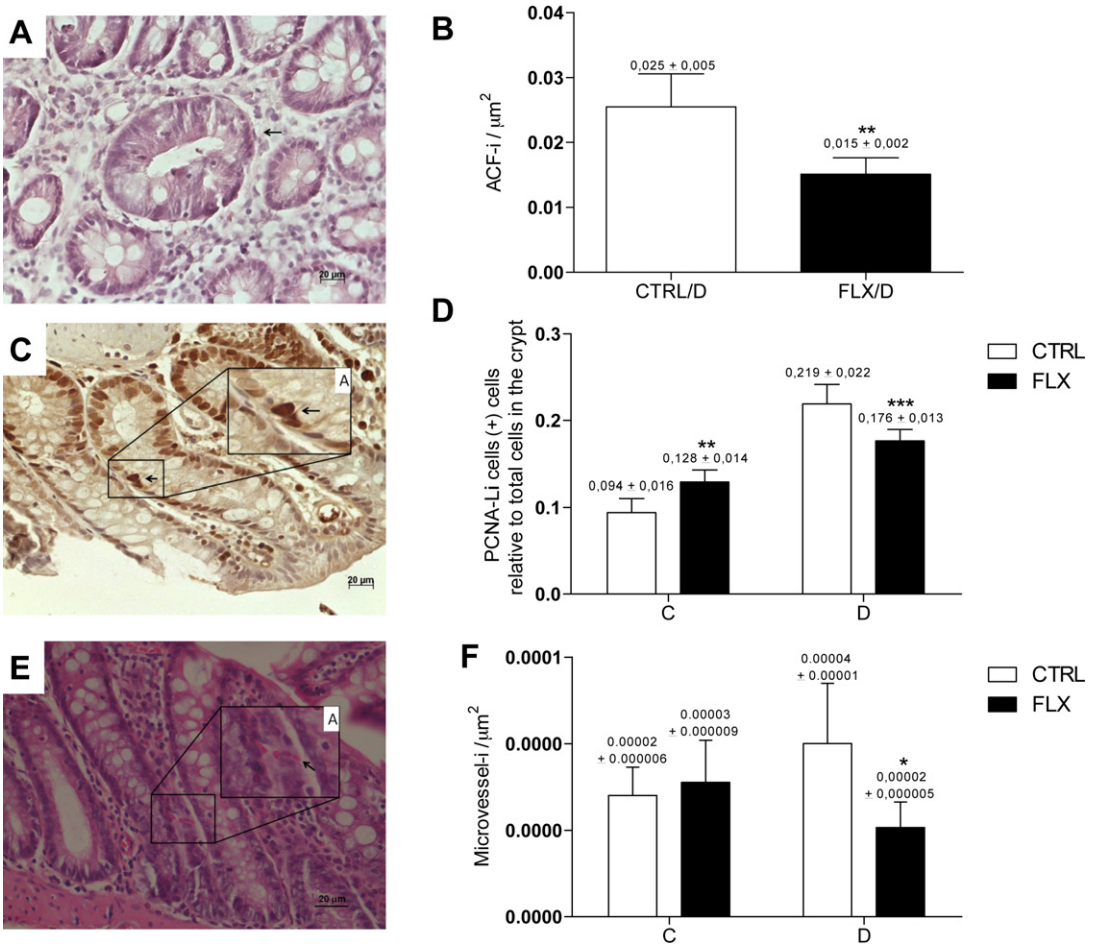

Fig. 2. (A) Dysplastic aberrant crypt foci (ACF) is pointed by the arrow. The figure shows elongated cryptal luminal opening, crowded and pseudostratified nuclei, less nuclear polarity, numerous mitoses and lower number of globet cells (magnification 400×; scale bars 20 μm). (B) ACF-i (index) was demonstrated per μm^2 of colon mucosa in animals treated with 1,2 dimethylhydrazine (DMH). *P* values were determined by Mann–Whitney test ($P < 0.01$ *vs.* CTRL/D). (C) Proliferative cellular nuclear antigen (PCNA) (+) cells are shown by the arrow (400×; scale bars 20 μm). (C.A) PCNA (+) cell is indicated by the arrow (high magnification). (D) Proliferative process in colonic crypt cells according to PCNA-Li (labelling index; $P < 0.01$ *vs.* CTRL/C; $P < 0.001$ *vs.* CTRL/D). (E) Colonic longitudinal sections reveal the presence of microvessels surrounding crypts and heading towards them (400×, bars 20 μm). (E.A) Microvessel towards crypt area is indicated by the arrow (high-magnification). (F) Microvessel-i (index) was demonstrated per μm^2 of colon tissue ($P < 0.05$ *vs.* CTRL/D). Bars and whiskers: mean ± SD (standard deviation). *P* values were determined by two-way ANOVA and Bonferroni post hoc test.

development of preneoplastic injuries is not well characterized. In this way, dysplastic ACF (Fig. 2A) and crypt proliferative activity (Fig. 2C) were decreased in carcinogenic FLX-treated rats ($P < 0.007$ and 0.001; Fig. 2B and D), despite its activity in the promotion of proliferation in non-carcinogen treated rats ($P < 0.01$).

3.3. *FLX controls over proliferation and COX-2 protein*

As previously shown (Liang et al., 2004; Waldner et al., 2010), dysplastic ACF development is also related to microvessels enlargement. Therefore, crypt surrounding microvessels (Fig. 2E) were reduced in carcinogenic FLX-treated rats ($P < 0.05$; Fig. 2F). Also, it decreased VEGF expression within PCCS (Fig. 3A) in DMH-treated rats ($P < 0.001$; Fig. 3B) and, reduced COX-2 expression (Fig. 3C) in non-DMH and DMH-treated groups ($P < 0.01$; Fig. 3D).

4. Discussion

In this study we demonstrated that FLX and its metabolite are present in the colon tissue and this treatment possibly increased 5-HT levels by decreasing SERT activity resulting in the suppression of 5-HIAA release. Thus, FLX was quickly diffused into multiples body-sites, as colon, due to its high lipophilicity (Lefebvre et al., 1999) and possibly blocked SERT-function (Gill et al., 2008), resulting in the imbalance of 5-HT metabolism (Bertrand et al., 2010). Despite the current knowledge that high 5-HT levels are implicated in the induction of cell proliferation and tumor growth (Arends et al., 1986), 5-HT selectively inhibited the colon adenocarcinoma growth by constricting tumor arterioles (Lubbe and Huhnt, 1994). Furthermore, FLX has been revealed as a great apoptosis inducer inhibiting tumor development (Arimochi and Morita, 2006; Lee et al., 2010).

Our analysis is driven by the hypothesis that besides FLX effect on the upregulation of 5-HT levels, their co-related activity possibly promoted the blockade of 5-HT2C receptors. On the other hand, endogenous upregulation in this amine levels seemed not to be correlated to the promotion of malignant crypt changes, as noticed by its metabolism and recognition. FLX and N-FLX have been shown to enhance the rate of desensitization in 5-HT-receptors (Brink et al., 2004; Choi et al., 2003), reducing both Na^+ and Ca^{2+} currents as a noncompetitive antagonism activity (Eisensamer et al., 2003). Also, 5-HT potentially desensitized 5-HT2C receptors after a short cell exposition to this amine (Briddon et al., 1998), and the blockade of 5-HT1 and 5-HT2-receptors subtypes inhibited tumor cell proliferation (Tutton and Barkla, 1980, 1986). Additionally, 5-HT treatment promoted tumor but not crypt cell proliferation (Tutton and Barkla, 1980), whereas colon tumor cells treated with sulforaphane revealed decreased 5HT1A, 5-HT2C, and SERT levels, suggesting a lower tumor progression (Mastrangelo et al., 2008).

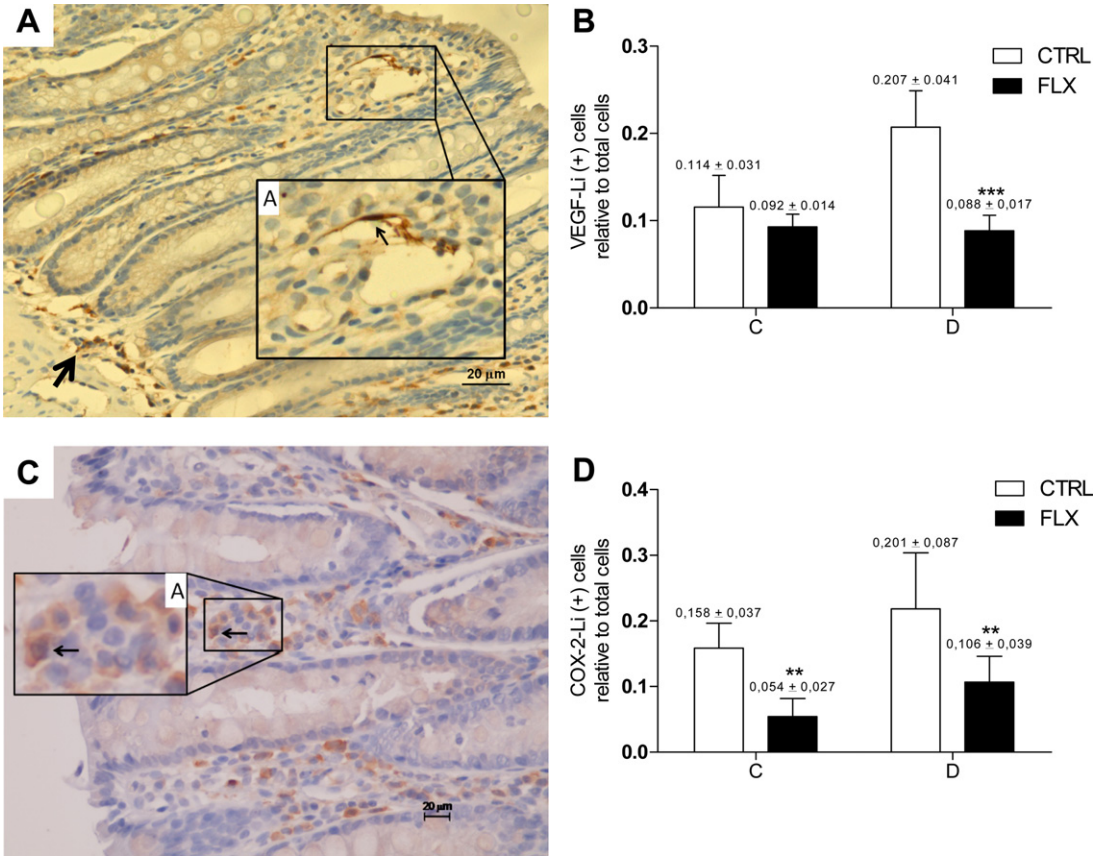

Fig. 3. (A) Vascular endothelial growth factor (VEGF) (+) cells are shown by the arrow (200×; scale bars 20 μm). (A.A) VEGF (+) cell at microvessel wall is indicated by the arrow (high magnification). (B) VEGF-Li according to labelling index of cells for anti-VEGF antibody ($P < 0.001$ *vs.* CTRL/D). (C) Cyclooxygenase-2 (COX-2) (+) cells within pericryptal colonic stroma (PCCS) (400×; scale bars 20 μm). (C.A) COX-2 (+) cell is indicated by the arrow (high magnification). (D) COX-2-Li according to labelling index of cells for anti-COX-2 antibody ($P < 0.01$ *vs.* CTRL/C and D). Bars and whiskers: mean ± SD (standard deviation). *P* values were determined by two-way ANOVA and Bonferroni post hoc test.

Although FLX greatly controlled dysplastic ACF development, the results regarding epithelia proliferation seemed to be conflicting between non-DMH and DMH treated rats that received FLX. Such perspective readily implies a potential and complex differentiating activity between non-malignant and malignant cells, as previously reported (Serafeim et al., 2003). Based on previous observations about 5-HT activity in crypt proliferative activity (Tutton and Barkla, 1980), this trend towards increasing crypt cells proliferation in FLX given rats seems to be not directly correlated to serotonin activity, since its metabolism and recognition (data not shown) were blocked and its endogenous upregulation did not promote malignancy among carcinogen-treated rats. Furthermore, this preventive FLX activity against the repopulation of colon tumors is possibly corroborated by the requirement of tumor cells to take up 5-HT before being stimulated by it (Barkla and Tutton, 1981; Tutton and Barkla, 1987). Tutton and Barkla reported that FLX decreased the tumor growth as well as, the crypt proliferative activity in animals under DMH-treatment (Tutton and Barkla, 1982), in a direct relationship with 5-HT-receptors blockade (Tutton and Steel, 1979). Stepulak et al. have shown that FLX diminished the proliferation of colon tumor cells *in vitro* by increasing the expression of cell cycle inhibitors p53 and p21 associated with the lower expression of cyclin D1 and A (Stepulak et al., 2008).

The present role of FLX in the control of dysplastic ACF and microvessels development, related to lower VEGF expression within PCCS, are also pointing that the endogenous upregulation of 5-HT levels has a potential activity against early malignant injuries. Whereas, previous reports were quite clear about the supply of tumors by preexisting host microvessels since their early development (Skinner et al., 1990) and, 5-HT-receptors are not only associated with the control of malignant proliferation, likewise implicated to tumor microvascular process (Froberg et al., 2009; Sulaiman et al., 2008). It seems reasonable that 5-HT applied intratumorally effectively constricted tumor microvessels (Huhnt and Lubbe, 1995), and 5-HT combined with bioactive substances decreased colon carcinoma development by lowering blood vessels density (El-Salhy and Sitohy, 2002; El-Salhy et al., 2003). In addition, FLX has previously been shown to decrease VEGF plasma levels in splenic lymphocytes in aged rats (Kubera et al., 2009).

According to our COX-2 protein expression data, we are suggesting that there is an interaction between FLX and serotonergic activity, possibly downregulating 5-HT-receptors among stroma cells. Jin et al. have shown that FLX strongly suppressed proinflammatory markers, such as COX-2 in neuronal cells (Jin et al., 2009) and also decreased proinflammatory properties in peritoneal macrophages, redirecting them towards anti-inflammatory activity (Roman et al., 2009). It has been reported that DOI (1-[2,5-dimthoxy-4-iodophenyl]-2-aminopropane) treatment activated 5-HT2C receptor, stimulating COX-2 mRNA and protein expression (Mackowiak et al., 2002). Moreover, 5-HT-receptors have been shown to activate cyclic adenosine monophosphate (cAMP) formation (Amireault and Dube, 2005), which in turn phosphorylates Src and subsequently activates ERK1/2 upregulating COX-2 activity (Mastrangelo et al., 2008). Previous researches have shown that overexpression of COX-2 and VEGF factors can support the development of colon cancer, establishing a link between inflammatory process and malignant angiogenesis (Liang et al., 2004; Waldner et al., 2010). Thus, antiangiogenic therapies have been

suggested as successful strategies to control malignant development (Wang et al., 2008).

Our collective data suggest that FLX is a remarkable oncostatic agent that acts against the development of dysplastic ACF possibly due to its inhibitory effect on malignant proliferation and angiogenesis. Therefore, FLX activity is possibly associated with high 5-HT levels, blocking the colonic serotonergic metabolism and recognition, as a possible adjunct-factor against the malignant changes. According to our present findings in colonic epithelia and PCCS, we believe that FLX might control the carcinogenic interaction between crypt cells and surrounding stroma elements, controlling microvessels development, VEGF, and COX-2 expression. Despite our results indicate that FLX may control preneoplastic development in colon tissue, further studies should be accomplished.

Conflict of interest

The authors have no conflicts of interest to disclosure.

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