Evaluation of the Extrapineal Sources of Melatonin in Patients with Lymphocytic Colitis

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Abstract

Research Article

Melatonin can be synthesized by different cells in the gastrointestinal tract. The aim of this study was to evaluate the expression of melatonin-synthesizing enzymes: arylalkylamine-N-acetyltransferase(AANAT) and N-acetylserotonin methyltransferase (ASMT) in colonic mucosa and urinary 6-sulfatoxymelatonin excretion(aMT6s) in patients with lymphocytic colitis(LC) related to the number of intraepithelial lymphocytes(IEL) and enterochromaffin cells (EC). The study included 32 healthy subjects (HS group) and 36 patients with lymphocytic colitis (LC group). The diagnosis was based on histological and immunohistochemical performances using mouse monoclonal antibodies (chromogranin A – LK 2H10). EC were counted in 10 fields of each bioptate at 40x magnification. The level of mRNA expression of AANAT and ASMT was estimated in colonic mucosa with RT-PCR. The urine concentration of aMT6s was determined by photometric method. Significant differences were found in obtained results between HS and LC group: the number of IEL 14.1±3.41 vs. 32.4±5.50 per 100 surface colonocytes (p < 0.001); the number of EC 32.2±10.1 vs. 70.4±21.2 (p < 0.001); AANAT -1.32±0.47 vs. 2.32±0.65 (p < 0.001; ASMT – 1.51±0.59 vs. 2.80±0.92 (p < 0.001) aMT6s- 13.4±4.87 vs. 19.9±6.29 (p < 0.001). Moreover, a positive correlation was found between the number of EC and ASMT (p = 0.011) and between EC and aMT6s (p < 0.001). No correlation occurred between the number of IEL and AANAT p = 0.932) and AMST (n = 0.536) and aMT6s (p = 9.288). These results indicate that the enterochromaffin cells in colonic mucosa are the main extrapineal source of melatonin in patients with lymphocytic colitis.

Keywords: Lymphocytic colitis, intraepithelial lymphocytes, enterochromaffin cells, melatonin synthesizing enzymes, 6-sulfatoxymelatonin

Introduction

Melatonin extrapineal synthesis occurs in many organs (1), but mainly in the gastrointestinal tract, where it plays an important protective role (2,3). This is associated with its many beneficial properties including antioxidant, antiinflammatory and immunomodulatory ones (4,5,6). Disturbances in the secretion of this hormone may cause functional and organic changes. Both decreased (7) and increased melatonin secretion was observed in functional bowel disorders (8.9). Different factors such as age and gender (10), clinical picture of the disease (11) and even diet (12) can affect the results. The number of melatonin-synthesizing cells is also significant as in the irritable bowel syndrome both the increase (13.14) and decrease of their density (15.16) was found in the colonic mucosa. The proliferation of enterochromaffin cells was more frequently observed in inflammatory diseases of this intestine (17, 18). Our previous studies proved that the number of EC cells depends on the clinical condition of the patients and their number increases mainly in the acute phase of ulcerative colitis (19). It was also found that in the acute phase of inflammation the increase in the number of EC cells does not result in increased urinary aMT6s excretion. Moreover, other researchers observed a reduction of melatonin level in patients with ulcerative colitis (21).

Subsequent studies in this group of patients demonstrated that despite the increase in the expression of enzymes synthetizing melatonin (AANAT, AMTS) in the colonic wall, the urinary aMT6s excretion was lower than in healthy subjects (22). In inflammatory foci melatonin was recognized to be metabolized also non-enzymatically ("consumed") in oxidative reactions. However, in patients with lymphocytic colitis (LC), both the expression of these

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enzymes and urinary aMT6s excretion was higher than in healthy subjects as well as in patients with active ulcerative colitis (22). These differences may result from different composition of inflammatory cells. An increase in the number of intraepithelial lymphocytes > 20/100 colonocytes is the basic criterion for the diagnosis of this disease. Lymphocytes are also believed to be the source of melatonin (23.24,25). In this sense, lymphocytosis would be a beneficial change, the excess of melatonin would protect the mucous membrane against erosion and ulceration. However, it is not clear why melatonin does not prevent chronic diarrhea which is the main symptom of lymphocytic inflammation.

The aim of the study is to evaluate the expression of AANAT and AMST and urinary aMT6s excretion in patients with lymphocytic colitis with reference to the number of intraepithelial and enterochromaffin cells in the colonic mucosa.

Patients and Methods

Patients

The study included 32 healthy subjects (control group -C, aged 38,6± 9,4 years), and 36 patients with lymphocytic (group LC, aged 44,1±12.2 years). The study was performed in the years from 2009 to 2017. The diagnosis was based on clinical, endoscopic and histological examinations. Only patients with inflammatory changes in whole colon were included in the study. The patients with LC had normal endoscopic picture, but intraepithelial lymphocytes went over 25 per 100 epithelial cells. Moreover, the patients had intensive and chronic symptoms such as non-bloody diarrhea, abdominal pain, fecal incontinence and others.

Exclusion criteria: small intestinal bacterial overgrowth, food intolerance, parasitosis, exocrine pancreatic deficiency, thyroid dysfunction, metabolic and mental diseases and chronic use of drugs except for 5-aminosalicylates.

Study Design and Procedures

The following routine laboratory tests were performed in all subjects: blood cells count, quantification of protein, glucose, bilirubin, iron, urea, creatinine, and thyroglobulin concentration and activity of alanine and asparaginine aminotransferase, alkaline phosphatase, gammaglutamyltranspeptidase, amylase, and lipase. Furthermore, serum concentration of C-reactive protein latex agglutination photometric assay-COBAS (by INTEGRA 800), fecal calprotectin (Sandwich ELISA -Quantum Blue Reader) and urine concentration of 6sulfatoxymelatonin (aMT6s) with enzyme immunoassay.

On the day of testing of urinary aMT6s excretion the subjects received only condensed liquid meals (Nutridrink 3x400 ml) with a total energy value of 1800 kcal and 1500 ml noncarbonated isotonic mineral water.

After completion of 24-hour urine collection, the urine was centrifuged and the samples were stored at -70° C. The urinary aMT6s concentration was determined using Immuno Biochemical Laboratories kit (No.RE 54031). The measurements were performed by photometry at wavelength of 450 nm(Expert 96-Reader – Biogenet,). The obtained results were converted from nanogram per mililiter to microgram/24 hours.

Biopsy specimens were collected from right, transverse and left colon. The number of the intraepithelial lymphocytes was determined using HE staining. To determine the number of enterochromaffin cells immunohistochemical method was used with mouse monoclonal antibodies (chromogranin A – LK 2H10, Cell Marque Co., Hague, The Netherlands) and Ultravision Quanto Detection System (HRP-DAB, Immunologic BV,Duiven, The Netherlands) in the range 10 fields in each bioptate at 40x magnification.

The level of mRNA expression was estimated with RT-PCR and 50 mg of colonic tissues were used for this purpose. Briefly, colonic tissues were rapidly permeated to stabilize and protect cellular RNA with RNA stabilization reagent RNAlater® (Quiagen, Hilden, Germany). Prior to total RNA isolation colonic tissues were homogenized with TissueRuptor (Quiagen, Hilden, Germany). Next, the total RNA was isolated using Quiagen RNeasy Plus Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's protocol. The quantity quality of isolated RNA were estimated and spectrophotometrically by Take3 plate on Synergy HT Microplate Reader (BioTek Instruments, Winooski, USA). The real-time gene expression analysis was performed using the TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, USA) with probes for AANAT (Assay Hs01063208_g1) and ASMT ID: (Assay ID: Hs00187839 m1) and SensiFASTTM Probe No-ROX One-Step Kit (Bioline, Taunton, USA). The HPRT (The hypoxanthine phosphoribosyltransferase, Assay ID: Hs01003267 m1) gene was a reference. Real-time PCR reaction was performed with BioRad CFX96 (BioRad, Hercules, CA) according to the suggested RT-qPCR conditions. Expression analysis was performed with CFX Manager 1.6 software (BioRad, Hercules, CA) using 22Ct method with HPRT gene as reference target

Results

The urinary excretion of 6-sulfatoxymelatonin was higher in patients with LC than in the control group -13.2 ± 4.79 vs. $19.4\pm6.20 \pm \mu g/24h$ (p<0.001,Table I).

Table I General characteristics the subjects included inthe study and laboratory and histological results: urine 6-
sulfatoxymelatonin excretion (aMT6_{s)}, number ofintraepithelical lymphocytes (IEL) and enterochromaffin
(EC) in colonic mucosa; **** p <0.001</td>

Features		Control group (n=32)	Lymphocytic colitis (n=36)
Age-years		38.6± 9.2	44.1±12.2
Gender	Men	16	16
	Women	16	20
aMT6 _{s -} µg/24h		12.9±4.80	19.6±6.25 ***
IEL		14.1±3.41	32.4±5.50 ****
EC		33.2±10.1	70.4±21.2 ***

The number of intraepithelial lymphocytes (IEL) in healthy subjects was $14.1\pm3,41$ and in patients with LC - 32.4 ± 5.50 per 100 surface colonocytes (p<0.001,Table I). Similarly, in patients with LC the number of enterochromaffin cells(EC) was statistically higher than in healthy subjects – 33.2 ± 10.1 vs. 70.4 ± 21.2 (p<0.001,Table I). Furthermore, $74,6\pm8.2\%$ enterochromaffin cells were located in the glandular area of the colonic mucosa.

The level of AANAT expression was 1.32 ± 0.47 in the control group and 2.32 ± 0.65 in patients with LC (p<0.001, Figure 1).



Figure 1. Mean relative level of arylalkylamine-Nacetyltransferase (AANAT) and N-acetylserotonin methyltransferase (ASMT) expression in colonic mucosa in healthy subjects (C) and patients with lymphocytic colitis (LC); *** - p< 0.001

In patients with LC the level of ASMT expression was also higher than in healthy subjects -1.51 ± 0.59 vs. 2.80 ± 0.92 (p<0.001, Figure 1).

No correlation was found between the number of intraepithelial lymphocytes and the level of AANAT expression (p=0.932, Figure 2) as well as ASMT expression (p=0.536, Figure 3).

A positive correlation was found between the number of enterochromaffin cells and the level of AANAT expression (p=0.064, Figure 4) and the level of ASMT expression (p<0.001, Figure 5).



Figure 2 Correlation between the number of intraepithelial lymphocytes (IEL) and the level of arylalkylamine-N-acethyltransferase (AANAT) expression in colonic mucosa in patients with lymphocytic colitis; p= 0.932



Figure 3 Correlation between the number of intraepithelial lymphocytes (IEL) and the level of Nacetylserotonin methyltranserase (ASMT) expression in colonic mucosa in patients with lymphocytic colitis; p = 0.536







Figure 5. Correlation between the number of enterochromaffin cells (EC) and the level of Nacetylserotonin methyltransferase (ASMT) expression in colonic mucosa in patients with lymphocytic colitis; p < 0.001

No correlation was fond between the number of IEL and urinary aMT6s excretion (p=0.288, Figure 6).





A positive correlation was found between the number of EC and urinary aMT6s excretion (p<0.001, Figure 7).





Discussion

The obtained results confirm previous observations indicating an increased secretion of melatonin in patients with lymphocytic colitis. They also confirm the opinion that enterochromaffin cells are the main source of extrapineal melatonin, although they do not exclude the participation of lymphocytes in the synthesis of this indoleamine.

The number of EC cells in patients with LC is much higher compared to healthy subjects. The proliferation of these cells in this type of colitis was also reported by other researchers (26, 27). These changes are probably the consequence of the increased expression of proinflammatory cytokines in the colonic wall.

The increase in the urinary excretion of the main melatonin metabolite (aMT6s) is an exponent of the total pool of secreted melatonin. Nevertheless, high activity of melatonin- synthesizing enzymes indicates a significant role of the intestinal fraction.

Melatonin, demonstrating paracrine and endocrine activity, can protect the colonic mucosa against the effects of toxic oxygen species and microcirculation disorders and thus counteract the colonic wall destruction. This protective effect of melatonin is not always sufficient as macroscopic changes may occur in some patients (28).

Aminosalicylates, glucocorticoids and immunosuppressants are used in the treatment of LC and other inflammatory diseases (UC, CD) but the therapeutic effects are often unsatisfactory. It is not known whether addition of melatonin supplementation could result in the improvement of the treatment efficacy. In general, the indication for supplementation is reduced secretion of this hormone, for example in postmenopausal women (29). Nonetheless, beneficial effects were obtained after melatonin supplementation in various diseases of the gastrointestinal tract. Its properties such as relaxing smooth muscles (30-33), analgesic (34) and lowering the patients' emotional tension (35) were used in the treatment of irritable bowel syndrome. The antiinflammatory and immunomodulatory properties were confirmed in the treatment of inflammatory diseases of the colon (36-40).

It is not clear why, despite the increased melatonin synthesis, its amount does not protect patients with LC against the occurrence of chronic diarrhea. This confirms the opinion that the pathogenesis of this disease is complex and not fully recognized (41-42). Cytokines and prostaglandins (PGE2), which affect the motor and secretory function of the intestines, play a significant role in the inflammatory process (43). Moreover, beside melatonin EC cells may produce other tissue hormones such as serotonin or motilin peptide YY (44) which stimulate the motor function of the intestines. The interactions between melatonin and serotonin have not been fully recognized, either. Melatonin may increase tissue serotonin concentration by stimulating the expression of tryptophan hydroxylase (45) and by inhibiting the reuptake of this neurotransmitter (46). The effect of melatonin on the rate of gastrointestinal transit is also controversial because besides its inhibition (47), diarrhea was observed during the treatment with melatonin (48).

The above discrepancies indicate the need for further research on the role of melatonin in chronic diseases of the gastrointestinal tract.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Written consent was obtained from each subject enrolled in the study and the study protocol was approved by the Bioethics Committee of Medical University of Lodz (RNN/242/06/KB).

Statistical analysis

The non-parametric Kruskal-Wallis test was used to evaluate the expression of TPH-1, AANAT and ASMT and urinary aMT6s excretion in three groups: C, UC and LC. The Mann-Whitney test was used for comparison of mean values. The correlation between the value of urinary aMT6s excretion and concentration of plasma CRP and fecal calprotectin was estimated by the determination of Pearson's correlation coefficient and linear regression equation and rang Spearman coefficient. The differences between the results were regarded as significant when a " P" value = 0,05 – 0,001. Statistica 9.0 (StatSoft, INC USA) and MS Excel @007 (Microsoft Co., USA) were used for statistical analysis.

Conclusions

These results indicate that patients with lymphocytic colitis may display high level of melatonin-synthesizing enzymes in colonic mucosa related to the increased melatonin synthesis. High synthesis of melatonin in LC possibly protect colonic mucosa against macroscopic damage.

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Author contributions

Chojnacki C conceived the study, screened the patients, and carried out the clinical procedures; Błasiak J

performed genetic procedures; Fichna J carried out the biochemical analysis; Chojnacki J participated in the study design; Popławski T participated in genetic procedures and interpreted the data. All authors wrote and approved the final version of the manuscript.

Conflict of interest: The authors declare no conflict of interest

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