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RESEARCH ARTICLE

Investigation of the effect of intracolonic melatonin gel formulation on acetic acid-induced colitis

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Abstract

The aims of the present study were to develop a colon-specific gel formulation of melatonin with sodium alginate and to evaluate its *in vitro* characteristics and intracolonic performance on oxidative stress parameters, such as nitric oxide (NOx), malondialdehyde (MDA) and glutathione (GSH) levels in rats with acetic acid-induced colitis. The melatonin-alginate gel formulations were prepared and their physico-pharmaceutical properties were determined. Formulation M5, which contained 3% of sodium alginate and 20% polyethylene glycol, was used for *in vivo* studies. The *in vivo* studies were conducted in rats with acetic acid-induced colitis. NOx, MDA and GSH levels were determined and histological investigations were performed. It was found that formulation M5 was the most suitable formulation for the colon-specific melatonin gel, in terms of pH, viscosity, drug release and mucoadhesion properties. The MDA levels in the tissues of Group 2 (treated with an intracolonic gel formulation without melatonin) were found to be significantly higher than in Group 1 (the untreated group). NOx levels decreased with the intracolonic and systemic melatonin treatment in the colitis-induced rats. Neither intracolonic nor intra-peritoneal (IP) melatonin treatment affected GSH levels. The epitelization of the colon tissues in groups administered with intracolonic melatonin, IP melatonin, and the intracolonic gel formulation without melatonin was much better than that found in the untreated group. It was concluded that melatonin participated in various defense mechanisms against the colonic inflammatory process, and that the dose, route and formulation type were the most important parameters in the effectiveness of melatonin.

Keywords

Antioxidant, glutathione, inflammatory bowel disease, malondialdehyde, nitric oxide, oxidative stress

History

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Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is a chronic inflammatory disorder of the gastrointestinal tract. The exact pathogenesis of the disease is not fully understood, although interaction between immune factors, genetic suspectibility and the environment seem to be the most important elements (Triantafillidis & Triantafillidis, 2009). Oxidative stress produced by free radicals is one of the risk factors of the disease. Oxidative stress arises when there is a marked imbalance between the production of reactive oxygen species (ROS) and antioxidant levels (Kruidenier & Verspaget, 2002). High free-radical levels and low antioxidant capability are well-known characteristics of IBD (Lih-Brody et al., 1996).

Melatonin (N-acetyl-5-methoxytryptamine), a multifunctional molecule, is synthesized from l-tryptophan in the pineal gland and involved in the control of circadian and photoperiodic systems in mammals. High melatonin concentrations are found in the gastrointestinal tract and enterochromaffin cells have been suggested as the source of its synthesis (Bubenik et al., 1998). It is known that melatonin and its metabolites are potent antioxidants, both as direct free radical scavengers and as indirect antioxidants (Tan et al., 1994; Reiter et al., 2007; Rybka et al., 2014).

As ROS play a significant role in the pathogenesis of IBD (Head & Jurenka, 2004), the antioxidant properties of melatonin may have a protective effect in colonic inflammation.

Various studies conducted with rodents have shown that melatonin administration reduces the severity of colitis. These effects were attributed to a variety of mechanisms, including inhibition of nitric oxide (NOx) production, inhibition of COX-2 expression, inhibition of NF-kappa activation, reduction of colon immunological injury through regulation of macrophage activity, reduction of proinflammatory cytokines, reduction of bacterial translocation, reduction of matrix metalloproteinase-2 and -9 activity, and modulation of signal transduction pathways and apoptosis (Terry et al., 2009).

Necefli et al. (2006) reported that melatonin administration reduced mucosal damage in rats that had 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. It significantly

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decreased the elevation of malondialdehyde (MDA) levels, while increasing the GSH in the colonic tissue of these rats. The mechanism of the protection associated with melatonin was attributed to its anti-oxidant, antiapoptotic and anti-inflammatory effects. Similarly, the intracolonic administration of melatonin attenuates the colon injury macroscopically and microscopically, reduced the myeloperoxidase activity (MPO), and ameliorated the degree of lipid peroxidation of the colon in the TNBS-induced colitis in rats (Mei et al., 2002).

It has been demonstrated that pre-treatment with melatonin reduces the signs of colonic inflammation induced by acetic acid in rats. This effect was independent of the route of melatonin administration, and similar results were obtained after IP and after intracolonic administration. It was explained that melatonin participates in various defense mechanisms against colonic inflammatory processes by preserving the important endogenous antioxidant reserve of glutathione (GSH), and by preventing lysosomal enzyme disruption through inhibiting enhanced MPO activity, thus reducing the extent of colonic damage, mainly in the early phase of colitis. It may be a promising therapeutic agent for ulcerative colitis (Nosál'ova et al., 2007).

Tahan et al. (2011) reported that intraperitoneally administered melatonin inhibited not only oxidant damage but also inflammatory cytokines, and improved the colonic inflammation induced by acetic acid in rats. It has a dual action as an effective anti-inflammatory and an antioxidant.

Mucoadhesive drug delivery systems work by increasing the duration of drug residence at the site of activity. Alginates are biodegradable, biocompatible and mucoadhesive polymers (Gombotz & Wee, 1998). Their mucoadhesive features may increase their utility as potential delivery vehicles for drugs targeted to mucosal tissues (Rajinikanth et al., 2003). The adhesion of an alginate gel to mucosal tissues localizes the drug and prolongs the duration of drug residence, thereby potentially improving the overall effectiveness.

The aims of the present study were to develop a colon-specific gel formulation of melatonin with sodium alginate and to evaluate its *in vitro* characteristics and intracolonic performance on oxidative stress parameters, such as NOx, MDA and GSH levels in rats with acetic acid-induced colitis. In studies evaluating the antioxidant efficacy, melatonin with an isotonic saline solution has been administered either intracolonically or through the IP route (Cuzzocrea et al., 2001; Mei et al., 2005; Marquez et al., 2006; Necefli et al., 2006; Nosál'ova et al., 2007). To our knowledge, no *in vitro* or *in vivo* intracolonic formulation study has been performed with melatonin. The present study aimed to prolong the residence time of melatonin by using an intracolonic administration of a gel formulation.

Materials and methods

Materials

The active ingredient melatonin (assay $\geq 98\%$) was obtained from Sigma-Aldrich Corp., St. Louis, MO, USA. It was stored at 2–8 °C. Sodium alginate (Protonal LF 10/60) was obtained from FMC Biopolymer, Philadelphia, PA, USA. The viscosity of the sodium alginate (1% in acetic acid) was measured with a viscometer (DV-III + Rheometer TC502, Brookfield, Middleboro, MA, USA) and found to be 650 ± 8 cP at 37 °C.

Other materials used for the *in vitro* and *in vivo* studies, such as polyethylene glycol 400 (PEG 400), sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate anhydrous, disodium hydrogen phosphate dihydrate and sodium chloride, were obtained from Merck, Darmstadt, Germany.

Animals

Wistar rats, male ($n = 28$), weighing 200–300 g were housed in clear plastic cages and fed with pellets and tap water.

In vitro studies

Preparation of the gel formulations

The sodium alginate type Protonal LF10/60 is used as a wound care agent (Hnin-Ei et al., 2012). Poly(ethylene glycol) (PEG) is an uncharged hydrophilic polymer. The PEGylation enhances the stability of drug delivery systems in digestive fluids and mucus, so that degradation is delayed (Lautenschläger et al., 2014). Based on this knowledge, alginate and PEG were chosen for the preparation of the gel formulations in this study.

Melatonin was mixed with PEG 400 for 5 min. A sufficient quantity of water and sodium alginate (2 or 3%) was added and mixed for 15 min. The mixture was kept in an ultrasonic bath for 5 min to discard air bubbles and a clear gel formulation was obtained. The composition of the gel formulations are given in Table 1.

The melatonin solution administered by the IP route was prepared in a sterile saline solution containing PEG 400 (4%), just before administration. After the melatonin was completely dissolved, the intraperitonal solution was filtered through a membrane filter (pore size 0.22 μm).

pH measurements

pH values of the gel formulations were measured with a pH meter (Schott CG 840, Mainz, Germany) at room temperature 24 h after preparation.

Measurement of viscosity

The viscosity of the gel formulations was determined with a viscometer at a temperature of 25.0 ± 0.5 °C (DV-III + Rheometer TC-502 Brookfield, Middleboro, MA, USA). The spindle speed was adjusted to provide the highest percent torque value in the 10–100% range, as recommended by the Brookfield instruction manual. Spindle Nos 52 and 20 rpm were used for measuring viscosity.

Table 1. Composition of the gel formulations.

Formulation	Melatonin (%)	PEG 400 (%)	Protanal LF 10/60 (%)	Water (%)
M1	0.33	4	2	93.67
M2	0.33	15	2	82.67
M3	0.33	20	2	77.67
M4	0.33	15	3	81.67
M5	0.33	20	3	76.67

Mucoadhesion measurements

TA-XT*Plus* Texture Analyzer (Stable Micro Systems, London, UK) equipped with a 5 kg load cell was used for the mucoadhesion test. The *in vitro* mucoadhesion testing method was based on the measurement of the force (N), work (N·mm) and work of mucoadhesion (mJ/cm^2) needed to detach a sample of rat colonic mucosa from a gel formulation.

Testing conditions and instrumental parameters had been determined in a previous study (Tugcu-Demiröz et al., 2013).

Drug release studies

In vitro release of melatonin from gel formulations was carried out by USP dissolution apparatus I at a rotating speed of 50 rpm in 500 mL of a pH 7.4 phosphate buffer solution at 37 ± 0.5 °C. A 2 mL sample of each formulation was placed in dialysis tubing (Dialysis bag: D-0405, Sigma-Aldrich Corp., St. Louis, MO, USA, pore size of 12 000 Da) after one end was tightly tied. The other end was then sealed to obtain a bag which was immediately put into a basket. At pre-determined intervals, samples were withdrawn and melatonin concentrations were assayed spectrophotometrically at 278 nm.

The release profile obtained was evaluated in terms of release kinetics by applying zero order, first order and Higuchi kinetics.

Stability studies of the M5 gel formulation

The M5 gel formulation was chosen for further *in vivo* studies, because of the results of its *in vitro* characterization. Therefore, stability studies were carried out on solely the M5 gel formulation under conditions of 25 °C/60% relative humidity and 40 °C/75% relative humidity for a period of 2 months. Samples were drawn at regular intervals for stability analysis.

In vivo studies

The experimental protocol was approved by the Local Ethical Committee of Gazi University (G.Ü.ET-09-009).

After an overnight fast, colitis was induced in the rats (under xylazine (10 mg/kg) + ketamine (100 mg/kg) anesthesia) by intrarectal administration of 1 mL of acetic acid solution (acetic acid 3% in saline solution) with a 8-cm long cannula. The acetic acid solution was retained in the colon for 30 s. Thereafter, the rats were kept for 10 min in a Trendelenburg position to avoid reflux. The gel formulations were administered 24 h after induction of colitis. The rats were kept for 15 min in a Trendelenburg position after the administration of the gel formulations.

After induction of colitis, the rats were randomly allocated into four groups with six animals per group as follows:

Group 1: Untreated for 7 days

Group 2: Treated with intracolonic gel formulation without melatonin for 7 days

Group 3: Treated with intracolonic gel formulation containing melatonin (10 mg/kg) for 7 days

Group 4: Treated with IP melatonin solution (10 mg/kg) for 7 days

The test groups were sacrificed 7 days after treatment. The distal 12 cm of the colon was excised. While some of the samples were kept at −80 °C, the others were fixed in formalin for histological examination. Another group (Group 0) which was sacrificed 1 day after the colitis had been induced by acetic acid was used for histological studies only.

The levels of MDA, NOx and GSH in the colonic tissues were measured.

Determination of NOx levels

The NOx levels, the stable end products of NOx of the tissue homogenates, were determined by the Griess reaction (Green et al., 1982; Miranda et al., 2001). The tissue samples (0.1 g) were homogenized in a phosphate buffer (0.1 M) and then centrifuged at 3500 rpm for 15 min. Equivalent amounts of $VaCl_3$ were added to the 0.20 mL of supernatant for the reduction of nitrate to nitrite by $VaCl_3$ and left to incubate for 30 min at 37 °C. Then the sodium phosphate buffer and a mixture of equal amounts of Griess I (0.2% naphthalene diethylamine dihydrochloride) reactant and Griess II (2% sulphanilamide in 10% phosphoric acid (H_3PO_4)) reactant were added. After incubation for 10 min at 37 °C, the absorbance values of the samples were measured at 540 nm by spectrophotometry.

Determination of MDA levels

The MDA level, as the last step of lipid peroxidation, was measured in the tissues spectrophotometrically (Casini et al., 1986). The tissue samples (0.2 g) were homogenized in 10% trichloroacetic acid (1:9) and centrifuged at 4000 rpm for 20 min. 0.75 mL of supernatant was added to the solution of 1% butyl hydroxyl toluene in ethanol and 0.67% thiobarbituric acid. The solution was then left to boil over a water bath. The absorbance was measured at 535 nm by spectrophotometry.

Determination of GSH levels

The reduced GSH levels were measured in the tissue samples. The tissue samples (0.2 g) were homogenized under ice-cold conditions with a 10% trichloroacetic acid (1:9) solution in a tissue homogenizer, and centrifuged at 4000 rpm for 20 min. 0.25 mL of supernatant was added to $Na_2HPO_4{\cdot}2H_2O$ and the DTNB solutions (0.4 mg/mL in 1% sodium citrate). The mixture was assayed spectrophotometrically at 410 nm, immediately after mixing (Aykac et al., 1985).

Histological investigations

Colonic mucosal tissues to be used in light microscope studies were fixed in 10% formalin. They were dehydrated in alcohol, cleared in xylene and embedded in paraffin blocks. Each section of tissue in the blocks was deparaffinated with xylene and rehydrated, before finally being stained with hematoxylin/eosin and examined under a light microscope.

Statistical analysis

The data were presented as a mean ± standard deviation (SD). Statistical analyses were carried out using the GraphPad InStat (GraphPad Software, Inc., La Jolla, CA, USA) program's one-way analysis of variance. Values of $p < 0.05$ were considered to be significant.

Results

pH and viscosity measurements

The pH and viscosity values of the prepared gel formulations are shown in Table 2. All of them were transparent and had a homogeneous structure. The pH values of the formulations ranged from 6.9 to 7.1 and were assumed to be suitable for colonic administration.

The rheologic properties of the gel formulations were also studied. All formulations showed pseudoplastic flow, presenting an immediate flow after stress application. An increase in the amount of sodium alginate in the gel formulations led to an increase in viscosity. The M5 gel formulation was found to have the highest viscosity value whereas M1 had the lowest viscosity value.

Mucoadhesion studies

The mucoadhesion test was performed to measure the adhesive strength of the gel formulations to the rats' colonic mucosa. The results obtained for maximum detachment force (F_{max}), work of adhesion (W_{ad}) and work of mucoadhesion (W_{mucoad}) are shown in Table 3. The highest values were determined in the M5 formulation that included sodium alginate (3%) and PEG 400 (20%).

In vitro drug release studies

The drug release profiles from the gel formulations are depicted in Figure 1 and the main release kinetic characteristics of the gel formulations are gathered in Table 4. All the formulations showed similar release profiles except for the M1 formulation. About 96–99% of drug was released from the M2-M5 formulations at the end of 6 h.

Stability studies

The results of the stability studies of the M5 gel formulation in two different test conditions are shown in Table 5. The prepared M5 gel formulation presented good stability in terms of viscosity and drug content. On the other hand, no macroscopical physical changes were observed during storage. It was observed that its viscosity value was varied, but its fluctuations remained within the limits of application. It was found that the M5 formulation was the most suitable formulation for a colon-specific MT gel in terms of pH, viscosity, release and mucoadhesion properties. Therefore, the *in vivo* efficacy of the M5 gel formulation was investigated in rats with the acetic acid induced colitis.

Table 2. Results of pH and viscosity values of the gel formulations.

Formulation	pH	Viscosity (cP)
M1	6.9	394.04
M2	7.1	421.83
M3	7.1	570.62
M4	7.1	1308.00
M5	7.0	1798.50

Table 3. Mucoadhesion values of the gel formulations using texture analyzer with rat colonic mucosa ($n = 6$).

Gel formulations	F_{max} (N) ± SD	W_{ad} (Nmm) ± SD	W_{mucoad} (mj/cm^2) ± SD
M2	0.223 ± 0.010	0.077 ± 0.010	0.060 ± 0.004
M3	0.275 ± 0.026	0.074 ± 0.008	0.061 ± 0.006
M4	0.178 ± 0.006	0.074 ± 0.008	0.061 ± 0.006
M5	0.362 ± 0.028	0.163 ± 0.020	0.122 ± 0.016

SD: Standard deviation.

In vivo studies

The main oxidative stress parameters were evaluated in the colonic tissue of the rats. Figures 2–4 show the MDA, NOx and GSH levels. It is known that a high level of MDA in tissues shows an increase of oxidative stress. Tissue MDA levels in Group 2 were found to be significantly higher than in Group 1 ($p < 0.01$). There was no statistically significant difference between Group 3 and Group 4 in terms of MDA levels of colon tissues ($p > 0.05$). It was observed that the NOx level, which is an indicator of oxidative stress, increased in colitis-induced rats, whereas it decreased after treatment with intracolonic and systemic melatonin. Neither intracolonic nor IP melatonin treatment affected the GSH level.

Histological studies

Figure 5 shows the histological appearance of the colonic tissues, under a light microscope. We observed a massive loss

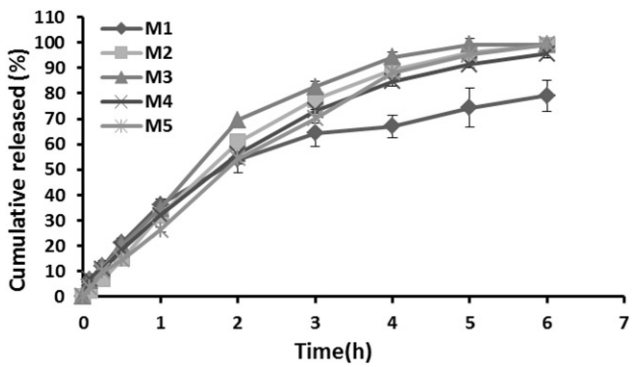


Figure 1. Release profiles of melatonin from gel formulations. The data represent the mean ± confidence interval, $n = 6$.

Table 4. Kinetic assessment of the release data of melatonin from gel formulations.

	Zero order		First order		$Q\sqrt{t}$	
Formulation codes	k_0	r^2	k_1	r^2	k	r^2
M1	12.0	0.894	0.343	0.712	34.9	0.979
M2	17.3	0.921	0.508	0.689	49.8	0.984
M3	16.9	0.894	0.410	0.736	49.0	0.970
M4	15.8	0.940	0.399	0.786	45.2	0.989
M5	17.2	0.954	0.474	0.763	48.7	0.987

k_0: zero-order release constant; k_1: first-order release constant; k: is the rate constant obtained from the slope of the linear regression of cumulative amount release per unit area versus square root of time; r^2: regression coefficient.

Table 5. The stability test results of the M5 gel formulation.

Time (week)	$t=0$	1	2	3	4	6	8
25 °C/60% Relative humidity							
pH	7.1	7.0	7.2	7.2	7.1	7.0	7.0
Viscosity (cP)	1497.7	1368.5	1327.6	1263.9	1101.9	1050.3	1046.4
Assay (%)	101 ± 1	100 ± 4	97.9 ± 4.9	99.6 ± 2.1	99.3 ± 2.6	99.5 ± 2.2	98.7 ± 0.6
40 °C/75% Relative humidity							
pH	7.1	7.1	7.1	7.2	7.1	7.0	7.1
Viscosity (cP)	1497.7	1244.2	1190.3	1154.3	1350.5	1344.7	1456.8
Assay (%)	101 ± 1	98.6 ± 2.9	97.8 ± 4.9	101 ± 0	97.4 ± 2.1	98.9 ± 3.8	98.0 ± 1.1

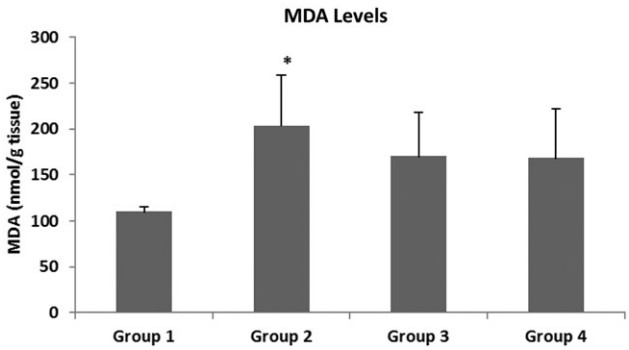


Figure 2. MDA levels in colonic mucosa. The data represent the mean ± SD, $n=6$ (*$p<0.01$, versus to Group 1).

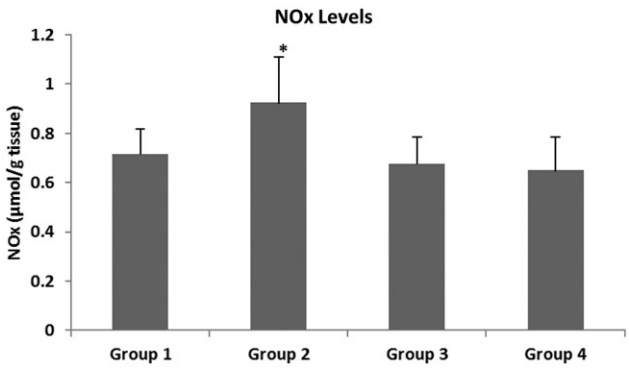


Figure 3. NOx levels in colonic mucosa. The data represent the mean ± SD, $n=6$ (*$p<0.05$, versus to Group 1 and Group 3, *$p<0.01$, versus to Group 4).

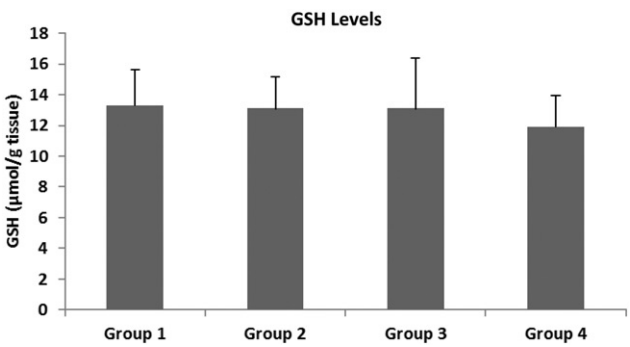


Figure 4. GSH levels in colonic mucosa. The data represent the mean ± SD, $n=6$.

of epithelium, severe inflammatory cell infiltration, distortion of intestinal crypt and mucosal and submucosal edema in the rats where colitis had been induced by acetic acid (Group 0, Figure 5A). In contrast, we found improvement in the structure of the intestinal crypt and inflammation, and disappearance of edema in other groups (Group 1–4, Figure 5B–E). Epitelization of the tissue in Groups 2, 3 and 4 was observed to be better than in Group 1.

Discussion

In vitro studies

Colonic gels with various amounts of sodium alginate and PEG were formulated in order to modulate their rheological and mucoadhesive properties. The results of preliminary experiments allowed for the optimal amounts of both polymers to be used (data not shown). The physicopharmaceutical properties of the prepared gel formulations were suitable in terms of pH and viscosity as well as in macroscopic appearance.

Due to its bioadhesive property and high viscosity, the gel formulation was expected to remain in the colon and to release the drug over a relatively long period of time, thus enhancing the therapeutic effect. Mucoadhesion tests were carried out to measure the adhesive strength of the gel formulations. The experimental parameters were determined by preliminary tests on the basis of the literature (Tugcu-Demiroz et al., 2013) and then they were validated. The bioadhesion of the M1 gel formulation containing the least amount of alginate and PEG could not be measured: this system displayed no adhesion. In comparison with the remaining formulations, it was found that the M5 formulation, comprising the highest amount of polymers, had the greatest mucoadhesion value (Table 3). The combination of both polymers contributed to an increase in mucoadhesion work. The binary system exhibited good adhesive properties.

The experimental technique was used for the *in vitro* release study of melatonin from gel formulations, which had emanated from a previously published method (Koffi et al., 2008). The results of *in vitro* release studies showed that about 79% of the drug was released during 6 h for the formulation M1, while a 95.6–99.3% release was obtained at 6 h for the other four formulations. Sodium alginate slowed down the *in vitro* release of melatonin. The developed formulations exhibited a sustained release of drug over a period of 6 h, thus increasing the residence time of the drug.

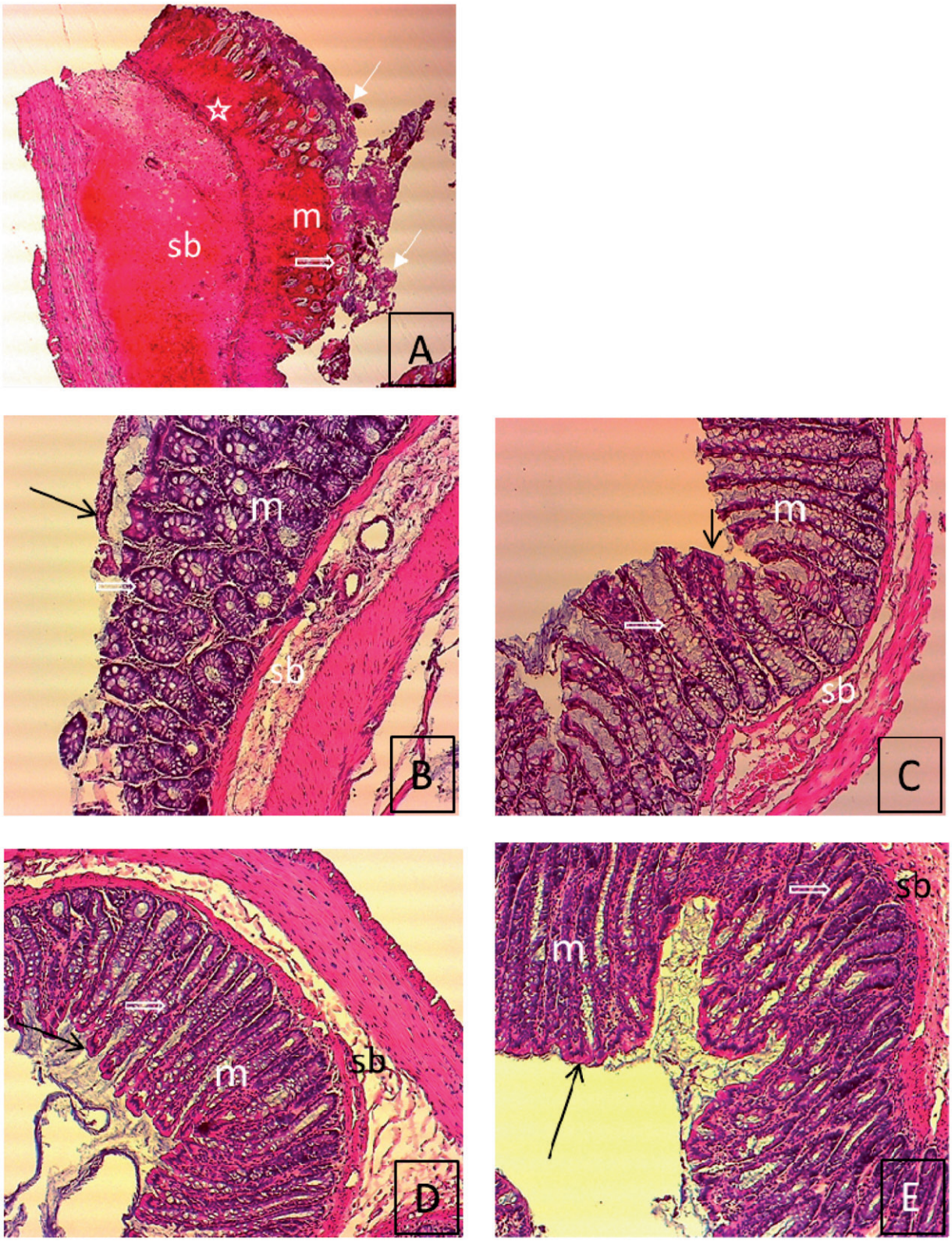


Figure 5. Histological features of rat colon sections in different groups. (A) Tissue image of group 0. (B) Tissue image of group 1, (C) Tissue image of group 2, (D) Tissue image of group 3, (E) Tissue image of group 4. Degeneration of epithelium and intestinal crypt, severe inflammatory cell infiltration, mucosal and submucosal edema in group 0 (A) (H&E. 4×10). Improvement in the structure of the intestinal crypt, incompleted epitelization, no edema in mucosa and submucosa in group 1 (B) (H&E. 10×10). Regular epithelium, improvement of the structure of the intestinal crypt, and no edema in mucosa and submucosa in Groups 2–4 (C–E) (H&E. 10×10). Epithelium (⟶), intestinal crypt (⟹), inflammatory cell infiltration (★), mucosa (m) and submucosa (sb).

The increase in the PEG ratio increased the release of melatonin from the gel formulations.

The data of percent released versus time were assessed for evaluation of the release kinetics. Upon checking the results according to r^2, it was found that $Q\sqrt{t}$ was the most suitable kinetic equation. The comparison of the release constants showed that the M1 formulation had the lowest values, whereas the others had higher values that were similar to each other. The kinetic assessment of release data revealed that the release of the active ingredient can be assumed to be diffusion-controlled.

The results of *in vitro* studies results showed that the M5 formulation was suitable for colonic administration in rats, according to its *in vitro* release profile, physicochemical and mucoadhesive properties. Moreover, it was assumed that this formulation was also appropriate in terms of stability. Therefore, further *in vivo* studies were performed with the M5 formulation.

In vivo studies

Melatonin is effective in the prevention or treatment of gastric or colonic injury, and it seems to participate in various defense mechanisms against colonic inflammatory processes (Tan et al., 2002; Nosál'ova el al., 2007; Triantafillidis & Triantafillidis, 2009; Tahan et al., 2011). While it has been suggested that melatonin acts as an agent protecting the gastrointestinal mucosa, the mechanisms of the protective effects of melatonin have not been fully elucidated. In the present study, a mucoadhesive alginate-PEG gel formulation containing melatonin was prepared. The effects of its performance on oxidative stress parameters, such as NOx which are the final stable products of NOx, MDA which is the final product of lipid peroxidation and the antioxidant GSH levels. These parameters were measured and evaluated in order to understand the effects of melatonin on oxidative stress in rats with acetic acid induced colitis.

Acetic acid was used in varying ratios from 2 to 8% in the literature (Fabia et al., 1992; Nosál'ova et al., 2007; Oruç et al., 2008). We applied various ratios of acetic acid (2, 3, 4%) to the rats and investigated the colonic tissues macroscopically and histopathologically after 24 h. After the evaluation of the macroscopic and microscopic data (not shown), it was decided that the 3% acetic acid ratio was suitable for the induction of colitis. Intrarectal administration of 3% acetic acid caused macroscopic damage of the colon. The colonic mucosa appeared hemorrhagic and ulcerated. The literature shows that a similar acetic acid ratio has been used in other studies for the induction of colitis (Millar et al., 1996; Tahan et al., 2011).

The melatonin doses administered to rats in various studies vary over a wide range from 2.5 mg/kg to 100 mg/kg (Li et al., 2005). The duration of treatment also varies widely and ranges from 48 h to 27 days (Terry et al., 2009). After review of the literature, a moderate dose (10 mg/kg) was chosen and administered to the rats for seven days in the present study. At the end of the treatment, colonic tissue samples were evaluated macroscopically. At the end of the seventh day, the macroscopic properties of all groups were observed to be similar. This situation was similar to the findings of Fabia et al. (1992).

Table 6. NOx, MDA and GSH levels in colonic mucosa (mean ± confidence interval).

Group	NOx (μmol/g tissue)	MDA (nmol/g tissue)	GSH (μmol/g tissue)
Group 1	0.715 ± 0.101	110 ± 5	13.3 ± 2.31
Group 2	0.924 ± 0.185	203 ± 56	13.1 ± 2.05
Group 3	0.676 ± 0.109	170 ± 48	13.1 ± 3.31
Group 4	0.648 ± 0.137	168 ± 54	11.9 ± 2.03

An increase in lipid peroxidation level is expected following injury, due to an increased oxidative stress. Our results were evaluated in terms of MDA, and no significant difference was observed between Groups 3 and 4 (Figure 2 and Table 6). This finding suggested that either IP or intracolonic administration of melatonin was not effective in scavenging the ROS in colon tissues. However, the MDA level of Group 2 (203 nmol/g tissue) was not significantly different from that of Group 3 (170 nmol/g tissue) either, but there was a reduced MDA level in Group 3. The decreased MDA level was found in the intracolonic melatonin administered group (Group 3). This finding showed that intracolonic melatonin administration has a protective effect on colon cells against increased lipid peroxidation. This decrease in MDA may have arisen due to administration time and the dose of melatonin.

Cells have been known to be hypersensitive to hypertonic media and to show an increased oxidative damage (Koziol et al., 2005). In the present study, colon cells encounter multiple stress conditions, such as acetic acid-induced colitis and hyperosmotic stress with intracolonic gel containing Na-alginate. This specific condition stimulated the generation of ROS. Increased ROS triggered lipid peroxidation in the colon cells in Group 2. We also suggested that the damaged cells in the colitis model may cause water loss due to the water-retaining properties of Na-alginate. Therefore, hyperosmotic stress could be increased in the damaged colon cells. This study demonstrated that a hypertonic medium (containing Na-alginate gel) increased the generation of superoxide and other reactive species in colon cells in Group 2. The combined effect of a hypertonic medium and a breakdown of the oxidant-antioxidant balance in the damaged cells may have affected the generation of superoxide and nitrosative reactive species, and therefore increased the lipid peroxidation of colon tissue.

Glutathione peroxidase and MDA levels of damaged tissues were investigated in a study conducted on 47 patients with ulcerative colitis and Crohn's disease (Tüzün et al., 2002). Similar to our study, no significant difference in MDA levels were observed. It was reported that free radicals were removed without causing lipid peroxidation.

It was observed that the NOx level, which is an indicator of oxidative stress, increased in our colitis-induced rats, whereas it decreased with the treatment of intracolonic and systemic melatonin, but this decrease was insignificant. However, a significant difference was determined between Groups 2 and 3 (Figure 3 and Table 6). The administration of the gel formulation with melatonin decreased the NOx level compared to that of the formulation without melatonin.

The NO level is at its highest in the inflammatory phase of the wound healing process. It is known that NO is produced by many different cell types in wounded tissue by three different isotypes of NOx synthase. Inducible NO (iNOS) is one of these isotypes. Fibroblasts, macrophages, keratinocytes and platelets produce NO by iNOS (Frank et al., 2002; Schwentker et al., 2002). When compared to the NOx levels of Group 1, the NOx levels of Group 2 were found to be significantly higher ($p < 0.05$). In other words, intracolonic Na-alginate administration without melatonin increased the NOx level in the colon cells. As mentioned before, in the presence of Na-alginate hyperosmotic stress could be increased in the damaged colon cells, which may affect the generation of superoxide and nitrosative reactive species. Intracolonic Na-alginate formulation may, therefore, have altered the colon tissue NOx levels in Group 2 (0.924 μmol/g tissue). There were no significant differences between the NOx levels of Group 3 (0.676 μmol/g tissue) and Group 4 (0.648 μmol/g tissue). This finding suggested that neither the IP administration of melatonin nor the intracolonic administration of melatonin changed the colon tissue's NOx levels. However, the NOx level of Group 2 was significantly different from those of both Group 3 and 4. These findings bring to mind two possible cases: the first of these is that, both intracolonic melatonin formulation and IP administration of melatonin were successful in reducing the NOx levels when compared to Group 2. The second of these is that, intracolonic melatonin administration has revealed the protective effect of melatonin in reducing the tissue NOx levels which increased in the presence of Na-alginate. Both intracolonic melatonin gel formulation and IP administration of melatonin decreased the levels of NOx in colon cells, and it is known that melatonin administration inhibits NOx levels due to its antioxidant properties. Our results showed similarity with the results of previously conducted studies (Mei et al., 2005).

GSH is a primary component of physiological systems protecting against free-radical damage (Casini et al., 1986; Tahan et al., 2011). In our findings, when considered in terms of GSH levels, a significant difference was not observed among the groups (Figure 4 and Table 6). GSH is an antioxidant itself, and local and systemic administration of melatonin had no effect on the level of GSH. This finding may suggest that other antioxidants, such as SOD, vitamin C and vitamin E may scavenge the generation of ROS or nitrosative species, and melatonin may act directly as a powerful antioxidant when there is no opportunity to use other antioxidants.

Although a wound healing effect of sodium alginate has been reported (Paul & Sharma, 2004), its positive effect on colon damage was not observed in the present study. Alginate gel showing oxidative effects itself significantly increased the lipid peroxidation and the NOx levels, thus hiding the protective effect of melatonin was hidden.

One of the important findings of this study was that there was a parallelism between altering tissue MDA and NOx levels in all groups (Figures 2 and 3).

When evaluated *in vivo* experiments and observations it can be said that interference in the colon via a canula can create an additional stress effect. In this case, the dose and the duration of treatment of melatonin were insufficient for such topical application. Unlike the situation with a wound incision, the integrity of the tissues was excessively disrupted in the acetic acid-induced colitis rat, and consequently, the use of melatonin by these cells was significantly decreased and the protective effect of melatonin could not be observed. It was thought that in such studies, pre-treatment with melatonin for a certain time, to raise the antioxidant capacity of the tissues before the induction of colitis, may be suitable.

Histological investigations concluded that the epitelization of the colon tissues in intracolonic melatonin, IP melatonin and intracolonic gel formulation without melatonin administered groups was much better than in the untreated groups. Improvement in the structure of the intestinal crypt and inflammation, and disappearance of edema were observed to be the same in groups which were untreated (Group 1), treated with gel without melatonin (Group 2), given intracolonic treatment (Group 3), and given systemic melatonin (Group 4).

Conclusion

It was concluded that melatonin participated in various defense mechanisms against colonic inflammatory processes, but the dose, route and formulation type were the most important parameters in establishing the effectiveness of melatonin.

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Declaration of interest

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