SUBCHRONIC EXPOSURE TO ACRYLAMIDE AFFECTS COLON MUCIN SECRETION IN JUVENILE WISTAR RATS

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Abstract: Acrylamide (AA) is an important industrial chemical worldwide. AA also forms naturally in many high-carbohydrate foods (bread, French fries, coffee, etc.) when they are heated. Since AA is ubiquitous in the human diet, and more than one-third of the calories we take in each day come from foods with detectable levels of acrylamide, the aim of this study was to determine the effect of subchronic AA treatment on colon goblet cell mucin secretion. Male Wistar rats were gavaged with AA for 5 days a week for 21 days. The animals were divided into three groups that were gavaged with different AA concentrations (0, 25, 50 mg/kg/day). Colon samples were processed for histochemical (PAS-AB, HID-AB) and immunohistochemical (anti-rat MUC2 antibody) staining to visualize mucins in the goblet cells. AA treatment showed an alteration in mucin production and secretion in that the amount of all investigated mucin types dropped. More prominent changes were detected in the upper crypt part part where a decreased number of goblet cell was observed. AA treatment elicited a significant reduction in neutral mucins, while acidic mucins showed linearly decreasing trend with respect to AA doses. Also, a linear reduction of MUC2 mucins was noticed. Sulfomucins were absent in the colon lower crypt part in all experimental groups, while in the upper crypt part both sulfo- and sialomucins were significantly decreased. The results of our study point to changes in the synthesis, differentiation and distribution of mucins after AA treatment, which can have adverse effect on colorectal health.

Key words: acrylamide; colon; goblet cells; mucins; juvenile rats

INTRODUCTION

For decades it was believed that the principal route of human exposure to acrylamide (AA) was occupational among industrial workers, but recent reports have shown that the general public is exposed to AA via cigarette smoking, drinking water and food consumption [1,2]. Current data have revealed that children's intake of AA could be as much as 2-3-fold higher than that of adults when expressed in body weight [3]. With increasing sources of the general public' exposure to AA, the need for understanding the toxicological risk associated with such exposure is in high demand. AA is a proven carcinogen in animals [4], and it is also known to be a neuro- and reproductive toxicant [5.6], but the only toxicological effect of AA established in humans is its neurotoxicity [7].

Gastrointestinal mucins produced by goblet cells play a significant role in the maintenance of mucosal homeostasis. Mucins consist of a peptide backbone containing glycosylated domains with O-linked glycans. O-linked carbohydrates can account for 80% of the molecular weight of mucins and they are often terminated with sialic acid or sulfo group [8]. Sialic acid is usually the terminal monosaccharide in the oligosaccharide chain of glucoconjugates; these terminal residues have a significant influence on mucus charge, mucus rheology and mucus degradation [9]. The apomucin backbone is coded by mucin (MUC) genes. More than twenty MUC genes have been identified and categorized [10], but MUC2 is the major secreted mucin expressed in the small and large intestine. Aberrant expressions of secreted mucins are found in some digestive diseases such as ulcerative

colitis [11] and other inflammatory bowel diseases [12] as well as intestinal infections caused by parasites, bacteria and viruses [13].

Mucins can be classified by their histochemical properties into neutral and acidic mucins, which are further categorized into sialomucins and sulfomucins [14]. In the normal colonic mucosa, the ratio of acidic to neutral mucins remains constant throughout the digestive tract [15]. The degree of mucin sulfation and sialyation in the matrix determine the mobility of secretory proteins through the meshwork pores, so the deregulation of mucin production can have serious health consequences [16]. In the proximal rat colon, sialomucins are the predominant content of the goblet cells all along the crypt epithelium, while goblet cells in the upper crypt part and surface epithelium may occasionally show sulfated material. The goblet cells of the upper crypt part and surface epithelium have a strong to moderate periodic acid-Schiff (PAS) reaction, whereas the mucus-secreting cells in the lower crypt part react weakly with PAS [17]. In the distal rat colon, goblet cells contain mainly sulfomucins, while sialomucins may be present in the upper crypt part. They also show a strong to moderate PAS reactivity. The histochemical characteristics of goblet cell mucins and their distribution in the distal rat colon crypt epithelium are similar to the human colonic mucosa [17].

Goblet cells can discharge mucins in response to a wide variety of stimuli, including nerve activation, inflammatory mediators and bioactive factors. The ability of goblet cells to respond to a broad range of intestinal insults by means of changes in mucin production and secretion indicates that these cells are important first-line defenders of the mucosa [15]. The physical state of the mucus, changes in the concentration of secreted mucins and strong dependence of its physicochemical properties on environmental factors such as ionic strength and pH, play an important role in many diseases [18].

Human exposure to AA through diet occurs throughout life, beginning *in utero*, since AA has been found to pass through the placenta [19]. Exposure continues in infants via breast milk [20] and baby food

[21] and lasts during the lifetime since numerous food items contain AA. When entering the gastrointestinal tract, the AA monomer pass the intestine cell monolayer by passive diffusion [22] and is rapidly absorbed [23]. Considering the aforementioned facts regarding mucin sensitivity to different stimuli, the aim of the present study was to investigate the effects of subchronic AA treatment on colon goblet cell mucin secretion in juvenile/peripubertal male Wistar rats.

MATERIALS AND METHODS

Experimental animals

Male Wistar rats, bred and raised in the Animal Facility of the Faculty of Sciences, University of Novi Sad, were used for the experiments. Animals were housed in plastic cages under laboratory conditions at 22±2°C and subjected to a controlled photoperiod (12 h light/dark cycle, lights on at 7 am). Pelleted food and tap water were provided *ad libitum*. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health. The investigation was performed with the permission of the Ethical Committee on Animal Experiments of the University of Novi Sad, Serbia (permission No IV-2010-01).

Experimental design

The experiment was performed on three animal groups with five animals per group, aged 23 days at the beginning of the study. The low-dose AA group received 25 mg/kg body weight per day of AA and the high-dose AA group received 50 mg/kg body weight per day of AA [24,25]. Acrylamide (Sigma-Aldrich Chemie GmbH, Taufkirchen Germany) dissolved in distilled water was administered orally in the morning (08:30-09:00 am). The control group received pure distilled water, also by gavage. Rats were gavaged five days per week during three weeks and killed 24 h after the last AA application.

Sample preparation

At the end of the experiments, the animals were decapitated under light ether anesthesia and colon-resected for further analysis. Proximal colon samples were fixed in 10% formalin, pH 7.0 for 24 h, dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin and cut into 5-µm-thick sections using a rotary microtome. To determine the general histochemistry of the mucins, the following staining procedures were used: periodic acid-Schiff-alcian blue (PAS-AB) (Merck, Darmstadt, Germany) reaction to characterize the neutral and acidic mucins, and high iron diamine-alcian blue (HID-AB) (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) staining to distinguish sulfated and nonsulfated (silylated) mucins. In the PAS-AB staining method, acidic mucins were stained blue and neutral mucin magenta, while a purple color was obtained when both neutral and acidic mucins were homogeneously present within the same goblet cell.

Immunohistochemistry

In order to estimate the expression of MUC2 mucins at the protein level in colon mucosa, an immunohistochemistry staining protocol was applied using the polymer detection system (Ultravision LP Detection System, Thermo Scientific, UK). High temperature antigen retrieval was carried out using 10 mM citrate buffer for 10 min in a microwave oven. The polyclonal rabbit anti-rat MUC2 (1:75) (Abcam, UK) in TBS (Tris-buffered saline) with the addition of 2% BSA (bovine serum albumin) (Sigma, Darmstadt, Germany) was applied to each section as a primary antibody. For visualization of bound antibodies, sections were incubated with 3,3'-diaminobenzidine (DAB) (Thermo Scientific, UK). Nuclei were counterstained with Mayer's hematoxylin.

Quantification

Semi-quantitative histological analysis was used to determine the effect of AA on the amount of mucins in the upper crypt part (UCP) and lower crypt part (LCP) of the colon (Fig. 1A). The numerical values from 0 to 5 (0 – absent, 1 – rare, 2 – small amount, 3 – moderate, 4 – high amount, 5 – large amount) correspond to an increasing mucin amount and were used for mucin approximations in all applied staining methods. The analysis was conducted on a light Reichert ocular microscope under a total magnification of 250x.

Statistical procedure

One-way analysis of variance (ANOVA) with the Fisher LSD (least significant difference) post-hoc test was used for the statistical analysis of the obtained numerical data. The p values less than 0.05 were considered significant. Statistical analysis was carried out using Statistica 12.6 (StatSoft Inc.)

RESULTS

In the colon, sections stained with PAS-AB a statistically significant decrease ($p<0.05$) in the distribution of goblet cells with neutral mucins was noticed in the LCP in the low-dose AA group compared to the control (Table 1, Fig. 1). A significant decrease ($p<0.05$) was also observed in the distribution of goblet cells containing a mixture of neutral and acidic mucin in the UCP in the high-dose AA group compared to both the control and the low-dose AA group. Goblet cells with acidic mucins in the UCP showed a linear increase in distribution (Fig. 2) with regard to the doses of AA compared to the control.

Regarding the ratio of neutral to acidic mucins in rat colon goblet cells (Table 2), a reduced distribution of neutral mucins to acidic mucins is evident in the UCP in both treated groups. This reduction is statistically significant ($p=0.0016$) only in the high-dose AA group. In the LCP, the ratio of neutral to acidic mucins in AA-treated groups decreased, but it was not statistically significant compared to the control group.

In rat colon sections stained with HID-AB (Table 3, Fig. 3), in the LCP sulfomucins were absent and a decreasing trend was noticed for all mucins in both

Table 1. Mean values with standard deviation for PAS-AB-stained mucins in goblet cells from rat colon mucosa after acrylamide treatment. Semi-quantitative analysis.

Experimental group and crypt region		Total mucins	Neutral mucins	Acidic mucins	Mixture of neutral and acidic mucins
CG	Upper crypt part	5.50±1.27	1.40±0.42	1.30±0.76	2.80±0.27
	Lower crypt part	5.80±0.45	0.50±0.35	4.40±0.22	0.90±0.22
LDG	Upper crypt part	4.90±1.39	0.80±0.67	1.40±0.42	2.87±0.85
	Lower crypt part	5.30±0.76	0.10±0.22 [a]	4.10±0.22	1.25±0.65
HDG	Upper crypt part	4.40±0.42	0.70±0.45	1.80±0.27	1.92±0.58 [a,b]
	Lower crypt part	5.30±0.57	0.20±0.27	4.20±0.45	0.83±0.26

CG – control group; LDG – low-dose group treated with 25 mg/kg AA; HDG – high-dose group treated with 50 mg/kg AA.
[a] $p < 0.05$ versus control group
[b] $p < 0.05$ versus low-dose group
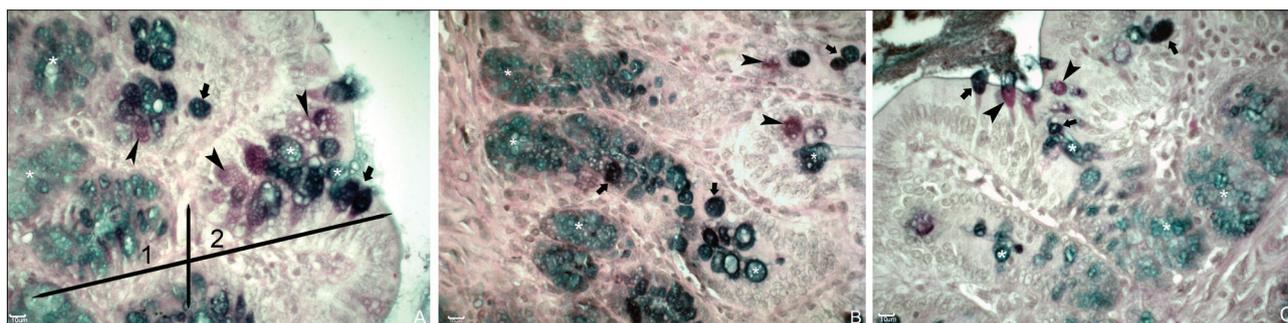For statistical analysis, Fisher's LSD post-hoc test was used

Fig. 1. Cross sections of rat colon. **A** – control; **B** – low-dose AA; **C** – high-dose AA. Lower crypt part (LCP) (1), upper crypt part (UCP) (2), acidic mucins (*), neutral mucins (arrow head) and cells with acidic and neutral mucins (arrow). Goblet cells with densely stained granules were observed along the crypt (A). In the LCP, a higher number of goblet cells was observed than in the UC; densely stained mucin granules were observed (B). Goblet cells with densely stained granules and shedding goblet cells were observed in the LCP, while in the UC we observed goblet cells at the moment of release of mucus granules; mucus material is present in the lumen (C).

Table 2. Ratio of neutral to acidic mucins and p values for this ratio in rat colon goblet cells after acrylamide treatment. Semi-quantitative analysis.

Exp. group	NM/AM ratio		p values for NM/AM ratio	
	UCP	LCP	UCP	LCP
CG	1.08	0.11	0.8028	0.0000
LDG	0.57	0.02	0.1281	0.0000
HDG	0.39	0.05	0.0016	0.0000

CG – control group; LDG – low-dose group treated with 25 mg/kg AA; HDG – high-dose group treated with 50 mg/kg AA; UCP – upper crypt part; LCP – lower crypt part.
NM – neutral mucins, AM – acidic mucins
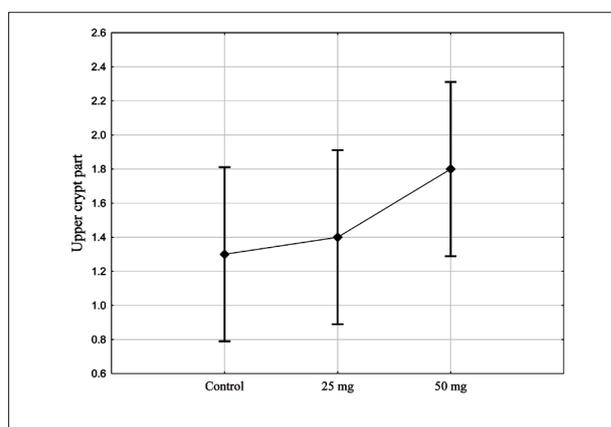For statistical analysis, Fisher's LSD post-hoc test was used.

Fig. 2. Distribution of goblet cells with acidic mucins in the upper crypt part of rat colon after acrylamide treatment.

Table 3. Mean values with standard deviation for HID-AB-stained mucins in goblet cells from rat colon mucosa after acrylamide treatment. Semi-quantitative analysis.

Experimental group and crypt region		Total mucins	Sialomucins	Sulfomucins
CG	Upper crypt part	4.60±0.22	2.60±0.22	2.00±0.35
	Lower crypt part	4.10±0.22	4.10±0.22	0.00±0.00
LDG	Upper crypt part	3.60±0.55 [b]	2.20±0.27 [a]	1.40±0.42 [a]
	Lower crypt part	3.80±0.27	3.80±0.27	0.00±0.00
HDG	Upper crypt part	3.40±0.65 [b]	2.10±0.22 [b]	1.30±0.45 [a]
	Lower crypt part	4.00±0.35	4.00±0.35	0.00±0.00

CG – control group; LDG – low-dose group treated with 25 mg/kg AA; HDG – high-dose group treated with 50 mg/kg AA.
[a] p<0.05 versus control group
[b] p<0.01 versus control group
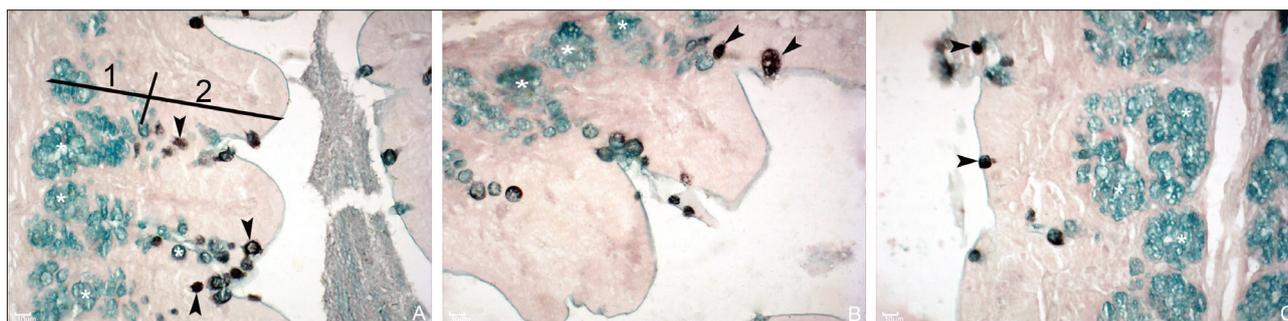For statistical analysis, Fisher's LSD post-hoc test was used.

Fig. 3. Cross sections of rat colon. **A** – control; **B** – low-dose AA; **C** – high-dose AA. Lower crypt part (LCP) (1), upper crypt part (UCP) (2), sialomucins (*) and sulfomucins (arrow head). In the LC, sulfomucins were absent in all groups (A-C), and a reduction of sulfo- and sialomucins in AA-treated groups (B, C) in the UC was observed. Sialomucins granules are densely stained while goblet cells with sulfomucins are shed or the granules are present in only one part of the cell (A-C). HID-AB staining. Scale bar: 10 μm.

Table 4. Ratio of sulfomucins to sialomucins and p values for this ratio in rat colon goblet cells after acrylamide treatment. Semi-quantitative analysis.

Exp. group	SuM/SiM ratio		p values for SuM/SiM ratio	
	UCP	LCP	UCP	LCP
CG	1.30	/	0.0125	0.0000
LDG	1.57	/	0.0072	0.0000
HDG	1.62	/	0.0072	0.0000

CG – control group; LDG – low-dose group treated with 25 mg/kg AA; HDG – high-dose group treated with 50 mg/kg AA; UCP – upper crypt part; LCP – lower crypt part.
SuM – sulfomucins, SiM – sialomucins
For statistical analysis, Fisher's LSD post-hoc test was used.

AA-treated groups. A statistically significant decrease in sialomucins (p<0.05 in the low-dose AA group; p<0.01 in the high-dose AA group), sulfomucins (p<0.05) and total mucins (p<0.01) were observed in the UCP in both AA-treated groups. The results of sulfo- to sialomucin ratio analysis (Table 4) demonstrated a dose-dependent reduction. In the LCP, the sulfo- to sialomucin ratio was the same as that in the control group.

The results of semi-quantitative analysis of MUC2-positive goblet cells in the proximal colon mucosa are given in Table 5 and Fig. 4. A linear reduction of MUC2 mucins was noted (Fig. 5) between groups in both crypt regions. In the UCP, a statistically significant decrease (p<0.05) of MUC2 mucins was noticed in both treated groups, while in the LCP significant changes (p<0.05) was observed only in the high-dose AA group.
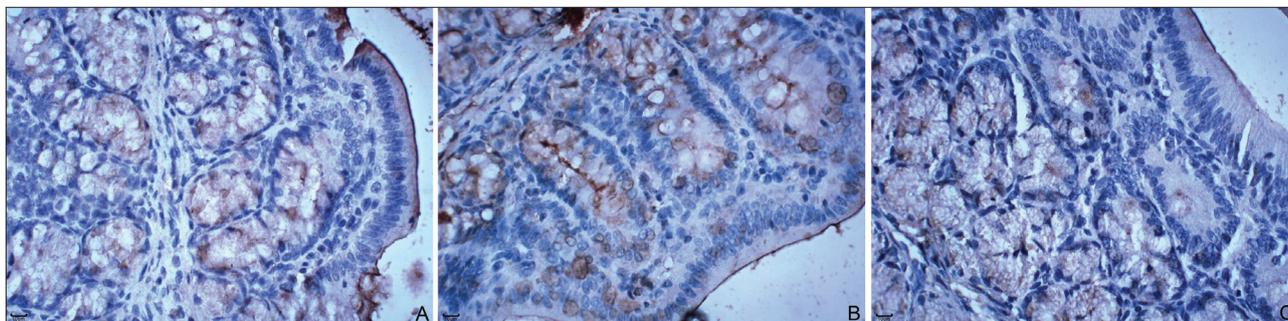
Fig. 4. Cross sections of rat colon. **A** – control; **B** – low-dose AA; **C** – high-dose AA. Lower crypt part (LCP) (1), upper crypt part (UCP) (2). Intensive expression of MUC2 along the crypt was observed (A). Moderate and intensive expression of MUC2 in the basal part of goblet cells was observed (B). Shedding of goblet cells was observed along the crypt (C). The apical surface of the colon epithelium is lined by the MUC2 mucin (A-C); the line is thickest in the control animals. Anti-MUC2. Scale bar: 10 µm.
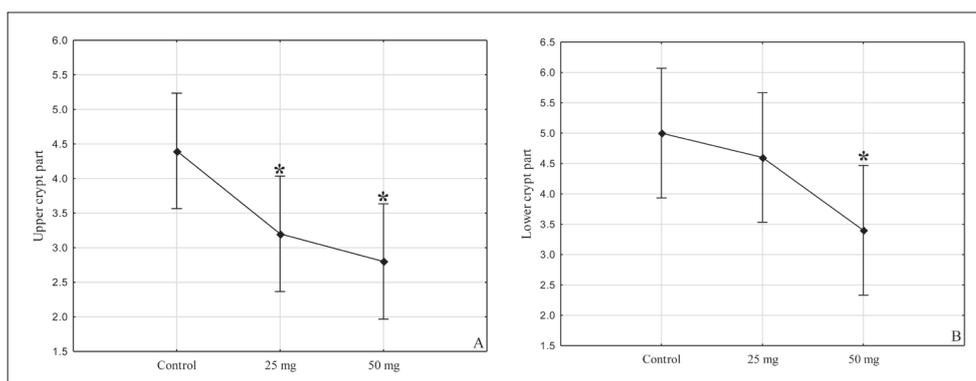

Fig. 5. Distribution of MUC2-positive goblet cells in the upper (A) and lower (B) crypt parts of rat colon after acrylamide treatment. * $p < 0.05$

According our results, AA caused a decrease in all investigated mucins. It reduced the synthesis of MUC2, the major colon secreted mucin, as well PAS-positive mucins, sialomucins and sulfomucins. AA affected the protein component of mucins and glycans linked to the peptide backbone.

DISCUSSION

The stability of the mucus layer, secreted by goblet cells, is essential in preserving the integrity of the intestinal epithelium. A disruption in intestinal homeostasis results in a defective mucus barrier with increased permeability that leads to inflammation and injury of the intestinal mucosal cells [26,27]. Multiple studies have shown that dietary factors may affect goblet cell numbers and mucin heterogeneity [28,29] and may modulate the secretory activity of goblet cells [30]. Chemical irritants, when applied to the luminal surface of the mucosa, have been shown to elicit local mucus release from goblet cells [31].

The MUC2 mucin forms a two-layered mucus system in the colon: the inner layer is anchored to the epithelium and devoid of bacteria and the outer layer is mobile and forms a habitat for the commensal flora [32]. As our results suggest, AA damaged the goblet cells' secretion mechanism and caused a reduction in the expression of MUC2 mucins. In the absence of MUC2 mucin, there is no inner mucus layer and bacteria reach the epithelial cell surface [33]. The relevance of this barrier is evident from studies in MUC2-deficient mice, which spontaneously develop inflammation [34] and intestinal tumors [35]. Similarly to our experiment, Lan et al. [36] showed that a high-protein diet also decreased the number of MUC2-positive and PAS-positive cells in colon mucosa.

Our results revealed that subchronic AA treatment increased the amount of acidic mucin in the colonic UCP, and decreased amount of neutral mucins in the LCP. Of major importance is the fact that the colon succeeded in maintaining the normal amount of acidic mucins under AA treatment, and in this way retained the integrity of the mucus protective layer, because it has been demonstrated that acidic mucins form a better barrier against bacterial translocation than neutral mucins [15]. The lower amount of goblet cells with neutral mucins could be associated with increasing maturity of the intestinal mucins in the presence of AA, since AA might decrease the dividing potential of goblet cells. In the small intestine it was shown that AA significantly decreased the number of proliferating cells and mitotic number in the crypt epithelium, whereas the apoptotic cell number was increased [37,38].

Chronic AA treatment [37] showed that the intestinal absorptive surface of mouse small intestine was significantly decreased by AA. Also, AA influenced the morphology of the crypts, which comprise the most metabolically active region of the intestine. Similarly, in the study of Tomaszewska et al. [38], AA led to a decrease in the number of goblet cells as well as decreasing the intact villi number and increasing the damaged villi number, which resulted in a decrease in the duodenum's absorptive surface. In the same study, the number of goblet cells in the jejunum increased, but the absorptive surface did not: conversely, it decreased like in the duodenum. El-Mehi et al. [39] reported that AA induced morphological changes in gastric mucosa in the form of loss of superficial epithelium, erosions and vascular congestion. They observed weak AB-positive goblet cells in the neck region and prominent AB-stained mucus in the basal part of the glands. According to these authors, the main cause of gastric mucosal alteration detected in this treatment was oxidative stress. Oxidative stress was also detected in human Caco-2 intestinal cells [40,41]. Since AA in our experiment decreased MUC2 expression, we assumed that AA caused oxidative stress in the goblet cells, disrupting normal cellular signaling.

Mucins are highly negatively charged due to the presence of the sulfo group or sialic acid [11]. The loss of these negatively charged monosaccharides from the oligosaccharide chain results in the reduction of negative charge from the secreted mucins, which is believed to be associated with colorectal disease [42]. Alterations in sialo- and sulfomucin chemotypes have been observed in patients with inflammatory bowel disease and colorectal cancer [43]. In our experiment, sulfomucins were absent and sialomucins showed domination in the LCP. Yusuf et al. [44] reported that the resistance of gastric mucosa to destructive agents may be dependent on the integrity of the sialic acids, and the desialylation of mucus may lead to the degradation of the mucus and eventually to the breakdown of the gastric mucus barrier.

We noticed in our previous work using the same experimental model, that subchronic AA treatment leads to a reduction of rat body weight [45]. The body weight of rats treated with 25 mg/kg and 50 mg/kg of AA continuously decreased in comparison to the control rats, whose body mass continuously increased. The reduction in body weight was more prominent in the group treated with 50 mg/kg of AA (p<0.001) compared to both the control and the low-dose AA group.

To the best of our knowledge, no studies have investigated the effects of AA on juvenile rat colon mucus production. On the basis of the present results, we conclude that AA treatment showed alterations in mucin production and secretion, but the main integrity of the mucus protective layer was preserved. Our results indicate that AA disturbed the balance between proliferation and differentiation of goblet cells, which is important for the homeostatic maintenance of colonic mucosa.

Authors' contributions: All the authors made a contribution to the concept of the experiment and its design. V.R., R.K. and I.K. conducted the experiment and collected the data. I.K. performed all the analyses, constructed the figures and wrote the manuscript. V.R. provided helpful points related to histological and statistical

analysis, and M.M. critically reviewed the manuscript and approved the version to be published.

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