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LOSS OF INTERSTITIAL CELLS OF CAJAL IN THE SMALL INTESTINE OF RATS WITH DIABETES MELLITUS

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Diabetic gastroenteropathy is a common complication in diabetes mellitus. Disturbance of interstitial cells of Cajal (ICC) distribution plays a significant role in the development of intestinal motility dysfunction.

The aim of this study was to investigate the alterations of the intramuscular and myenteric ICC in the small intestine of rats with diabetes mellitus.

Male Wistar rats were used and diabetes was induced by streptozotocin-nicotinamide (STZ-NA) application. The small intestine specimens were exposed to c-Kit antibody to investigate the ICC. Morphological changes of the cells were quantified by the numerical areal density of intramuscular ICC, and the ICC score of myenteric ICC. Results showed loss of ICC and their network in the small intestine in the diabetic group.

In conclusion, a statistically significant decrease in the number of intramuscular ICC and myenteric ICC was observed in all examined parts of the small intestine in rats with diabetes mellitus. Diabetes mellitus significantly changes the microenvironment of ICC and most likely the reduced signaling by insulin affects ICC and causes their loss.

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Key words: *interstitial cells of Cajal, diabetes mellitus, small intestine, gastroenteropathy*

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Introduction

Diabetes mellitus (DM) is a chronic systemic disorder of glucose metabolism characterized by hyperglycemia. Complications of diabetes include a broad group of disorders resulting from vasculopathy and neuropathy caused by hyperglycemia and oxidative stress. Diabetes affects almost all parts of the gastrointestinal tract (GIT), but the exact prevalence of diabetic gastroenteropathy is unknown, and the main reason for this is that gastroenteropathy symptoms are often attributed to other causes and accompanying diseases (1). There are data in the literature that complications of gastrointestinal motility, such as abdominal discomfort, gastroparesis, nausea and vomiting, diarrhea, slowed intestinal transit and constipation, occur even in 50 – 70% of patients after a ten-year illness (2, 3). Recent studies have shown that neuropathy is not the only cause of enteric gastroenteropathy, but that the loss of interstitial cells of Cajal (ICC) plays a significant role in the development of intestinal motility dysfunction (4). Complex and multifactorial mechanism of diabetic gastroenteropathy includes changes and loss of autonomic nervous system nerve fibers, myenteric plexus glia cells, smooth muscle cells and ICC (5).

A diverse group of intestinal cells in the digestive tract are responsible for the establishment and adequate functioning of intestine motility (6, 7). Based on different patterns of distribution, localization, morphological and functional characteristics, ICC are classified into several subtypes: myenteric ICC (ICC-MY) form a cellular network around the myenteric plexus between the circular and longitudinal muscle layers; intramuscular ICC located within circular (ICC-IMc) and longitudinal (ICC-IML) muscle layer; submucosal (ICC-SM) located at the border between the submucosal connective tissue and the inner muscle layer, septal (ICC-SEP) localized within the connective tissue septa surrounding the muscle bundles (8). The most important cells for maintaining intestinal peristalsis are ICC-MY and ICC-IM cells, which play the role of pacemaker determining the amplitude and frequency of slow waves, inhibitory neuromodulation and act as stress receptors and sensory transductors (9, 10).

Unlike numerous studies related to diabetic gastroparesis (11 – 15), studies that evaluate the

ICC in intestinal dysfunction in diabetes are rare, primarily due to the difficulties of the available methods of diagnosis (muscular wall of the intestine is not involved during routine biopsy of the intestine, and it is also difficult to reach the appropriate anatomical locations endoscopically) (16, 17). The effect of diabetes on the small intestine and colon motility has not been sufficiently studied, although an increased prevalence of constipation and diarrhea has been observed in patients with DM. Initial studies indicated that intestinal transit duration is slowed in animal models of DM, leading to bacterial overgrowth and consequent diarrhea (18), while other studies have shown the presence of accelerated intestinal transit in experimental models (19). This accelerated intestinal transit has been attributed to autonomic neuropathy and DM-induced denervation of sympathetic nerve endings. Prolonged intestinal transit time and constipation are common in patients with DM (1).

The aim of the present study was to identify distribution of intramuscular and myenteric ICC in the small intestine of rats with streptozotocin/ nicotinamide induced diabetes mellitus.

Materials and methods

Animal model

For this study small intestine sections from male Wistar rats (10 weeks old, weighting 230 – 250 g) were used. Experimental protocol used in this study was created in accordance with the National Guide for the Care and Use of Laboratory animals (Serbian Academy of Sciences and Arts, Serbia) and with the Rulebook for handling laboratory animals (Faculty of Medicine, University of Niš, Serbia), and approved by the Ethics Committee of the Faculty of Medicine, University of Niš, Serbia (permit number 12-519/7). Research was performed as a part of the Internal Project no. 38/20 of Medical Faculty of Niš at the Research Center of Biomedicine and Department of Histology and Embryology.

During the experiment, rats randomly divided into control (C) and diabetic (D) groups, were housed in plastic cages, within a controlled environment (constant air ventilation, humidity and temperature of 20 °C ± 2 °C, 12 h light/dark cycle and limitless access to food and water). Diabetes mellitus was induced by the combined usage of intraperitoneal injection of nicotinamide (Sigma Aldrich, USA) at a dose of 110 mg/kg in saline solution and intraperitoneal injection of streptozotocin (Sigma Aldrich, USA) at a dose of 45 mg/kg in ice-cold 0.1 mol/l citrate buffer (pH 4.5) according to the modified model described by Masiello et al. (20). Three and seven days after streptozotocin/nicotinamide (STZ-NA) administration, hyperglycemia was verified in the D group, using glucose meter Accu-check Performa (Roche Diagnostics, USA). In compliance with standard diabetes diagnostic criteria, animals with glucose level above 8.3 mmol/l were considered diabetic, while the animals, with glycemia results below the specified threshold, were excluded from D group. There were 10 animals in each of the C and D groups. At the same time commercially available rat enzyme-linked immunosorbent assay (ELISA) kit using rat insulin as the standard (Mercodia, Upsala, Sweden; catalog number 10-1250-01) was used to measure serum insulin levels. After six weeks from STZ/NA administration, the animals were sacrificed via exsanguinations through the bilateral thoracotomy in deep anesthesia (ketamine hydro-chloride, 100 mg/kg body weight). During the whole experimental period, animal body weight was monitored once a week after the overnight fasting and on the day of sacrifice, along with the daily monitoring of food and water intake.

Tissue preparation

Immediately after the sacrifice, the entire gastrointestinal tract of the animals was dissected in a block via an abdominal incision. Of this, the samples of small intestine (duodenum, jejunum and ileum separately) were specifically removed, treated and washed with saline solution. The obtained samples were fixed in 10% buffered formalin for 24 hours, after which they were paraffin-embedded using standard histological procedure and sequentially sectioned. 4 – 5 μm thick sections were stained using routine Hematoxylin and Eosin (HE) method, and c-Kit immunohistochemistry.

Immunohistochemistry

The slides were deparaffinized (at 58 °C degrees with xylene) and rehydrated in descending series of ethanol (100%, 96%, 70%) and distilled water, after which antigen retrieval solution, 45 minutes at 95 – 98 °C, using the EnVision Flex visualization kit (DM 828, 50x, Dako, Denmark) was applied. After the three rinses in distilled water, 3% hydrogen solution was used for 10 minutes. Primary antibody, Rabbit monoclonal anti-c-Kit (CD117) antibody (Abcam, Cambridge, UK, Ab32363 -dilution 1:100), was incubated overnight at 4 °C. The slides were then treated with secondary antibodies, (EnVisionTM FLEX High pH, code number K8000, Dako, Denmark) for 45 min at the room temperature. The resultant immune complexes were visualized using the Daco REAL EnVisionTM Detection System (Dako, Denmark). The slides were counterstained with hematoxylin, dehydrated in ascending series of alcohols (70%, 96%, 100%), and cleared with xylene.

Qcantitative Image Analysis

All slides were analyzed using an Olympus BX50 light microscope equipped with a Leica DFC

295 digital camera (Leica Micro-System, Reuil-Malmaison, France) with magnification at x200.

The microphotographs for quantitative image analysis were obtained by systematic random sampling method on Olympus BX50 light microscope equipped with a digital camera Leica DFC 295 digital camera (Leica Micro-System, Reuil-Malmaison, France). Numerical areal density analysis (NA) of intramuscular ICC, i.e. average number of cells per mm^2 of the circular and longitudinal muscle layer, was determined using ImageJ software (National Institute of Health, Bethesda, MD, USA; http://imagej.nih.gov/ij/). The cells were counted manually in order to avoid c-Kit positive mast cells which have different characteristics from ICC, like their shape, granular content and localization.

The assessment of myenteric ICC was done by estimating encirclement percentage of the ganglion by the processes of ICC-MY, i.e. ICC score (MP-sore), the semiquantitative method described by Den Braber-Ymker (21).

Analysis of the data obtained using the software SPSS Statistics (version 20, SPSS, Chicago, USA) was performed. The obtained data values were interpreted and compared using the Kruskal-Wallis test with Mann-Whitney U post hoc test.

Results

Establishment of the diabetes mellitus rat model

Glycemia values two hours after feeding in the D group (12.18 ± 0.73 mmol/L) were significantly higher ($p < 0.001$) compared to the control group (6.38 ± 0.61 mmol/L). Further, serum insulin values showed significantly lower values in the D group (192.15 ± 20.17) than in the C group (219.04 ± 18.9). There was no significant difference in body weight ($p > 0.05$) in the D group compared to the control group, however, moderate polydipsia and polyphagia were observed in the D group rats. Glycemia values two and eight hours after feeding, as well as body mass at the end of the experiment are shown in Table 1.

Table 1. Average values of glycemia 2 and 8 hours after a meal, serum insulin values and body weight at the end of the experiment

parameters	group	*N*	*X*	*SD*
2 h glycaemia (mmol/L)	Control	10	6.38	0.61
	D-group	10	12.18	0.73
8 h glycaemia (mmol/L)	Control	10	4.76	0.19
	D-group	10	6.73	0.38
Serum insulin levels (pmol/l)	Control	10	219.04	18.9
	D-group	10	192.15	20.17
Final body weight (g)	Control	10	397.50	11.38
	D-group	10	406.67	20.15

N-number of exp. animal, X-mean, SD-standard deviation

There was no difference in the thickness of the muscle wall between groups and also, no signs of necrosis, apoptosis, or infiltration by neutrophils or lymphocytes observed in all samples of the small intestine of the D group. On the histological sections of the small intestine, four layers were observed, going from the lumen to the surface: mucosa, submucosa, muscular layer consisting of circular and longitudinal sublayer and serosa. On immunohistochemical preparations stained with c-Kit, two types of c-Kit immunoreactive cells were observed, spindle-shaped or stellate multipolar ICC and oval mast cells with a large round nucleus without cytoplasmic appendages (Figure 1).

Distribution of c-Kit immunoreactive ICC subtypes of small intestine in the control and D groups are shown in Figure 1. Intramuscular ICCs of the circular muscle layer, on cross-sections, extended between smooth muscle cells, parallel to their axis. These cells were elongated spindle-shaped, with two long cytoplasmic processes starting from opposite ends of the cell body. On most of the cytoplasmic extensions of c-Kit immunoreactive cells of the circular layer, their branching could be observed, and some of the processes were connected to the corresponding extensions of neighboring cells. These cells were mostly single, scattered throughout the thickness of the circular muscle layer. On the longitudinal sections of the outer muscular layer of the GIT, no secondary branches of the ICC cytoplasmic extensions were observed, nor were their extensions in contact with neighboring cells. These ICC-IM cells were single and sparsely scattered throughout the longitudinal muscle layer.

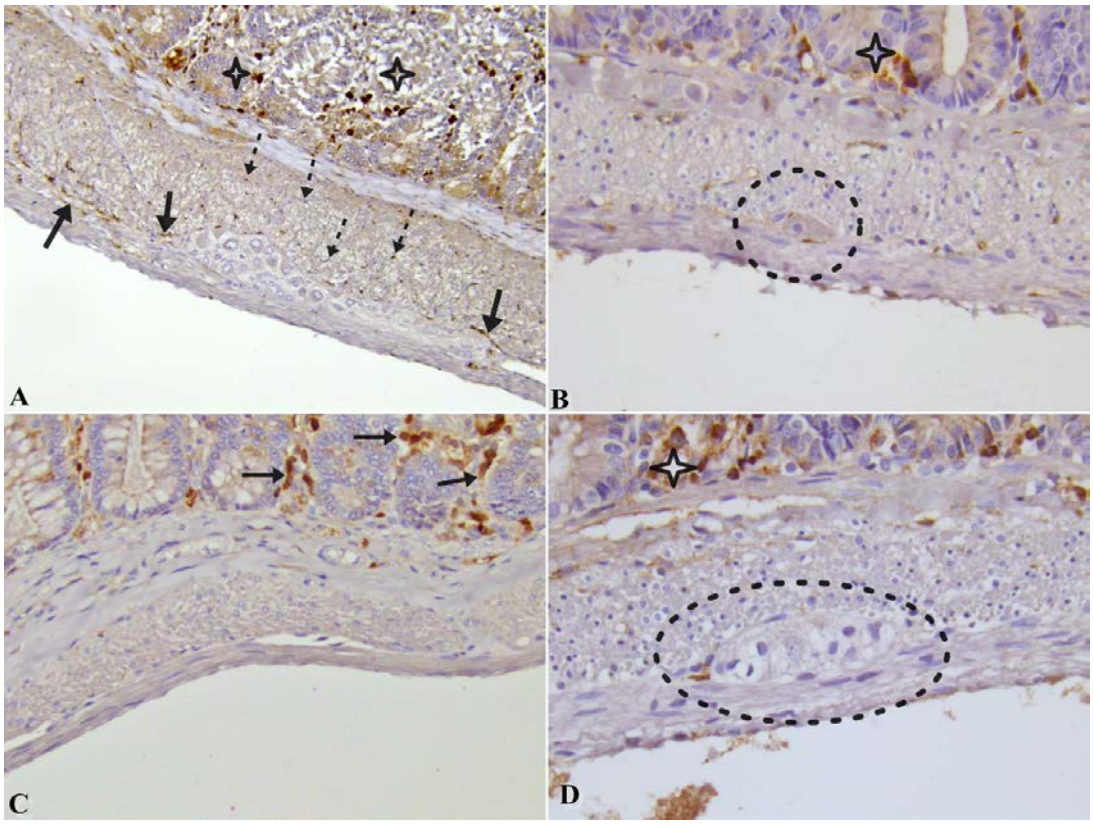

Figure 1. C-Kit immunohistochemistry of the small intestine of control (A, B) and D (C, D) group. A) on the cross-section of the jejunum, c-Kit immunoreactive mast cells are present in large numbers in the mucosa (asterisk); ICC-IM are located between smooth muscle cells, dominantly in the circular muscle sublayer (dashed arrows); ICC-MY are located around the MP ganglion, but do not completely surround it (arrow). B) on the cross-section of the ileum, ICC-MY are located around the MP ganglion (dashed circle); mast cells are present in the submucosa (asterisk). C) cross section of the jejunum; c-Kit immunoreactive mast cells are present in the mucosa and submucosa (arrow); in the smooth muscle layer there are no c-Kit immunoreactive ICC-IM and ICC-MY. G) cross section of the ileum; one c-Kit positive ICC-MY can be seen around the MP ganglion (dashed circle), c-Kit positive mast cells in the mucosa (arrow), c-Kit positive ICC-IM are rare in the muscle layer. A, C x 200; W, D x 400.

In the area of the myenteric plexus, star-shaped, multipolar c-Kit immunoreactive cells, which correspond to ICC-MY, were observed. These cells have long processes that connect and their appendages completely surround the edges of the ganglion of the myenteric plexus (Figure 1B).

Both types of ICC, with the same morphological characteristics as in the control group, were present in the D group. In contrast to the control group, ICC-IM were rare (Figure 1C, D). ICC-MY were also present in the D group, but much less frequently (Figure 1D) and did not completely surround the ganglions of the MP. MP ganglions with no c-Kit immunoreactivity in their surroundings were observed.

The average NAICC and the NA of intramuscular ICC subtypes values in the duodenum, jejunum and ileum of both the D and the control groups are shown in Table 2. The NA values are significantly lower in the D group ($P < 0.001$), compared to the control, as well as the NAICC values of the ileum in relation to the duodenum and jejunum within the control group.

Table 3 shows the average values of the degree of MP ganglion encirclement, i.e., ICC-score of the control and D groups. Testing the average values of ICC-score showed a statistically significantly lower density of MP-score ($P < 0.001$) in the D group compared to the control group in all samples of the small intestine.

Table 2. Numerical areal density (NA) of intramuscular ICC of the circular (ICC-IMc) and longitudinal (ICC-IML) muscle layer of the rat small intestine in the control and D groups

Small intestine region	parameter	group	*N*	*X*	*SD*	*CV*	*Mann-Whitney*
duodenum	N_A ICC	control	105	125.28	28.58	22.81	Z=9.943
		D-group	105	67.04	33.79	50.41	* P=0.000
	N_A ICC-IMc	control	105	106.37	33.82	31.80	Z=11.852
		D-group	105	34.60	18.59	53.71	* P=0.000
	N_A ICC-IML	control	105	10.16	4.50	44.29	Z=6.489
		D-group	105	5.85	3.55	60.66	p=0.000
jejunum	N_A ICC	control	102	120.72	40.03	33.16	Z=5.584
		D-group	102	88.03	37.65	42.78	* P=0.000
	N_A ICC-IMc	control	102	91.21	30.59	33.54	Z=11.296
		D-group	102	27.98	22.46	80.29	* P=0.000
	N_A ICC-IML	control	102	14.13	4.88	34.50	Z=5.978
		D-group	102	9.80	4.79	48.89	* P=0.000
ileum	N_A ICC	control	102	107.54	28.91	26.88	Z=5.793
		D-group	102	78.32	39.23	50.09	* P=0.000
	N_A ICC-IMc	control	102	80.79	21.57	26.70	Z=11.547
		D-group	102	26.00	19.43	74.73	* P=0.000
	N_A ICC-IML	control	102	10.66	5.88	55.10	Z=2.705
		D-group	102	8.63	4.50	52.19	* P=0.007

N – number of analyzed visual fields, X – mean value, SD – standard deviation, CV – coefficient of variation, NA-numerical areal density – the average number of cells per mm2 of the circular and longitudinal muscle layers. * – statistical significance

Table 3. Myenteric interstitial cells of Cajal (ICC-MY) score in the muscle layer of the rat small intestine in control and D group

Small intestine region	group	*N*	*X*	*SD*	*CV*	*Mann-Whitney*
			MP-score (%)			
duodenum	control	109	52.11	16.89	32.41	Z=9.031
	D-group	103	22.72	20.40	89.78	* P=0.000
jejunum	control	103	49.81	13.93	27.97	Z=8.510
	D-group	103	23.50	19.78	84.16	* P=0.000
ileum	control	107	53.36	16.76	31.42	Z=10.702
	D-group	103	18.26	16.76	91.79	* P=0.000

N – total number of evaluated ganglions, X – mean value, SD – standard deviation, CV – coefficient of variation, * – statistical significance

Discussion

The STZ-NA is widely used as an animal model of diabetes that corresponds to diabetes mellitus in humans, and is characterized by mild non-fasting hyperglycemia and slightly decreased insulin levels. Benefits of this animal model are that animals do not require exogenous insulin to survive and live longer so complications of diabetes can be analyzed (22). In addition, this model of diabetes proved to be suitable for examining the morpho-functional changes of ICC in the absence of complications of diabetes such as neuropathy and the effect of nerve fiber loss on ICC by nicotinamide dosage, and experimental duration (23).

Obtained results showed that ICC-IMc density is significantly higher in the circular muscle layer compared to the density of ICC-IML in the longitudinal sublayer in all small intestine regions. This distribution of ICC-IM is similar to the described distribution of ICC in the fetal and adult GIT of humans and rats (8, 24). The fact that smaller regions of tissue provide pacemaker activity for larger sections of circular and longitudinal muscles could explain the differences in the density of circular and longitudinal ICC-IM. Namely, Connor (25) observed in experimental studies that the separation of the circular from the longitudinal muscle layer of the small intestine inactivates the circular muscle, while the isolated longitudinal muscle retains its activity. The authors concluded that slow waves of peristalsis arise in the longitudinal layer and are amplified and propagated through ICC-IM. Also, muscle thickness of circular sublayer is higher than the outer longitudinal one and therefore a higher density of ICC-IM is needed for multiplication and propagation of the electric wave of peristalsis.

Intramuscular ICC of the small intestine are not elongated, spindle-shaped and densely distributed like ICC-IM of the stomach and large intestine (15, 23). In the small intestine, ICC-IM are rarely distributed and it seems that they do not cross-link. These results are consistent with the findings of Horiguchi and Mazet of ICC distribution in the GIT of guinea pigs and dogs (26, 27). Analysis of NA values of intramuscular ICC showed that the number of ICC significantly decreases in the small intestine, from the pylorus to the ileum, compared to the stomach and colon (23). The differences in the number of ICC-IMc of the small and large intestine can be explained by the fact that the smooth muscle cells of the colon, which are not well connected to each other by gap junctions, receive nerve signals through a rich network of ICC-IM, i.e., here ICC help "electrical" communication between smooth muscle cells. In the small intestine, smooth muscle cells of the circular layer are extremely well interconnected by gap junctions, which was shown by Seki and Komuro (28) by analyzing the expression of connexin 43 in the smooth muscle cells of the GIT in the guinea pig. Such mutual connection of smooth muscle cells enables the smooth muscle cells in the small intestine to function as a kind of syncytium, and accordingly, here ICC-IM are less numerous because they do not establish connections with each individual smooth muscle cell. Also, in the small intestine preganglionic vagal neurons innervate smaller groups of certain myenteric neurons, in contrast to the upper parts of the GIT where numerous connections with myenteric neurons are established. The differences in innervation by parasympathetic preganglionic fibers are reflected in the fact that the central nervous system has a much greater direct influence on the upper (esophagus and stomach) and most distal (sigma and rectum) parts of the GIT, and much less directly controls the functions of the small intestine and colon (29, 30).

In the small intestine, the average values of the areal numerical density of ICC-IM are statistically significantly lower in the ileum, compared to the duodenum and jejunum. The assessment of the ICC-score showed that there is no difference within different locations of the small intestine. The results of this study show that the number of intramuscular ICC and myenteric ICC is significantly lower in the group with diabetes. A similar loss of ICC has been observed in the small intestine and colon of experimental animals with diabetes (31, 32). Yamamoto et al., using the db/db model of type 2 DM, showed a reduced number of ICC in the small and large intestine (14).

The mechanism of ICC loss in diabetes has not yet been sufficiently investigated. The causes of the loss of these cells could be due to hyperglycemia and accompanying oxidative stress, reduced insulin levels and the absence of steel factor (stem cell factor), which is secreted by smooth muscle cells from the ICC environment under the influence of insulin and insulin-like growth factor (6, 13). Steel factor is c-Kit receptor ligand, and is necessary for the development and survival of ICC. In the absence of steel factor these cells transdifferentiate into smooth muscle cells or fibroblast like cells (32).

Shimoima et al. (33) and Suzuki et al. (34) showed decrease in c-Kit positive cells around the myenteric twelve hours after ischemia-reperfusion injury of the small intestine in rats, and also recovery of c-Kit positivity four days after. Chang and colleagues (35) reported that after experimentally induced partial occlusion of the ileum of mice, there is a decrease in the number of ICC-MY, proximal to the occlusion site, with disruption of the ICC network and consequent intestinal dysmotility. The release of pro-in-flammatory cytokines and chemokines has a negative effect on the number and function of nearby ICC, leading to damage to their network and dysfunction (36). With the absence of harmful factors, the number of ICC can recover and re-establish their network. Two mechanisms of ICC renewal have been proven. One is the differentiation from a small group of cells with weak c-Kit immunoreactivity (CD 117+), and pronounced CD 44+, CD 34+ immunoreactivity, which may represent a source of precursor cells for ICC renewal (37). Another mechanism is proliferation, and there is evidence that steel factor, NO from neurons, serotonin (via the 5HT28 receptor) and heme oxygenase-1 can induce proliferation of ICC (38). It is the potential of ICC restoration that may represent a potential therapeutic approach in diabetic enteropathy that should be further investigated.

Identifying cellular biomarkers may help us in developing better strategies for the diagnosis and management of the diabetic enteropathy. ICC as biomarkers could be linked to the treatment outcome and prognosis in these patients.

Conclusion

In conclusion, a statistically significant decrease in the number of intramuscular ICC and myenteric ICC was observed in all examined parts of the small intestine in rats with diabetes mellitus. Diabetes mellitus significantly changes the microenvironment of ICC and most likely affects the reduced signaling by insulin, thereby affecting the loss of ICC.

Acknowledgments

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GUBITAK INTERSTICIJALNIH ĆELIJA KAHALA U TANKOM CREVU PACOVA SA DIJABETESOM MELITUSOM

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Dijabetička gastroenteropatija predstavlja čestu komplikaciju dijabetesa melitusa. Poremećaj zastupljenosti intersticijalnih ćelija Kahala (IĆK) ima značajnu ulogu u patogenezi intestinalnih poremećaja motiliteta.

Cilj ove studije bio je da odredi distribuciju intramuskularnih i mijenteričnih IĆK u tankom crevu pacova sa dijabetesom melitusom.

Dijabetes melitus izazvan je streptozotocinom i nikotinamidom na animalnom modelu Wistar pacova muškog pola. Imunohistohemijsko ispitivanje vršeno je c-kit antitelom za identifikaciju IĆK. Zastupljenost intramuskularnih IĆK određivana je numeričkom arealnom gustinom, dok je za procenu mijenteričkih IĆK korišćen ICC-skor. Rezultati studije pokazali su smanjenje broja IĆK i njihovog umrežavanja u tankom crevu u grupi pacova sa dijabetesom.

Statistički značajan gubitak broja intramuskularnih i mijenteričnih IĆK zapažen je u svim uzorcima tankog creva pacova sa dijabetesom melitusom. Dijabetes značajno menja mikrookolinu IĆK i, najverovatnije, smanjenom signalizacijom preko insulina utiče na njihov opstanak.

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***Ključne reči**: intersticijalne ćelije Kahala, dijabetes melitus, tanko crevo, gastroenteropatija*