Histol Histopathol (2017) 32: 639-647

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

The effect of bilberries on diabetes-related alterations of interstitial cells of Cajal in the lower oesophageal sphincter in rats

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Summary. Diabetic gastroenteropathy involves not only the parasympathetic and sympathetic autonomic nerves, but also enteric neurons, smooth muscle cells and interstitial cells of Cajal (ICC). ICC are the cells of mesenchymal origin that occur within and around the muscle layers in the gastrointestinal tract. The objective of the present study was to investigate the alterations of ICC in the lower oesophageal sphincter (LOS) of streptozotocin-nicotinamide non-insulin-dependent diabetes rats. Moreover, we investigated possible ICC in rats with the same type of diabetes, treated with bilberry fruit extract, bearing in mind that its hypoglycemic effect had been already proven.

Male Wistar rats (10 weeks old) were used, and diabetes was induced by an intraperitoneal injection of streptozotocin, immediately after intraperitoneal application of nicotinamide. The specimens were exposed to anti-c-kit antibodies to investigate the distribution of ICC, and the smooth muscle cells were immunohistochemically labelled using anti-desmin antibodies.

Intramuscular ICC were very abundant in the LOS of rats. They were spindle-shaped, with two long processes connecting them into long linear sequences. In the LOS of diabetic rats, intramuscular ICC were rarely present and linear cell-cell connections between these cells were completely missing. In groups treated with bilberry, the number and distribution of ICC were exactly the same as in the above described rats with induced diabetes.

In summary, a decrease of intramuscular ICC, discontinuities and breakdown of contacts between ICC were observed in streptozotocin-nicotinamide induced diabetes rats and in groups treated with bilberry. Bilberry fruit extract was shown to have hypoglycemic activity, but without any protective effects on ICC in the LOS of diabetic rats.

Key words: c-kit, Gastroenteropathy, Peristalsis, Immunohistochemistry

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DOI: 10.14670/HH-11-829

Introduction

Gastrointestinal motility disorders related to enteric neuropathy occur in up to 30–50% of patients after 10 years of type I or II diabetes (Vinik et al., 2003; Boulton et al., 2005; Sfarti et al., 2010). Diabetic gastroenteropathy is multifactorial and involves not only the parasympathetic and sympathetic autonomic nerves but, within the gut, also enteric glia and neurons, smooth muscle cells, endothelium of capillary adjacent to the myenteric ganglia, mucosal endocrine cells and interstitial cells of Cajal (ICC). The pathogenesis of diabetic gastrointestinal neuropathy and gastroparesis has been the focus of recent reviews (Ordog, 2008; Ordog et al., 2009; Bagyanszki and Bodi, 2012).

ICC are the cells of mesenchymal origin that occur within the muscle layers and around myenteric plexus

(MP) in the gastrointestinal tract. These cells express CD117 (c-kit), a membrane receptor with tyrosine kinase activity (Ward, 2000; Takaki, 2003), and they depend on signaling via Kit receptors for their development and phenotype maintenance (Torihashi et al., 1995; Beckett et al., 2007; Radenkovic et al., 2010a). Certain subtypes of ICC form networks that play a role in gastrointestinal motor control and represent the source of slow-wave pacemaker activity (Huizinga and Lammers, 2009; Hwang et al., 2009), while other ICC subpopulations serve as mediators of enteric motor neurotransmission (Ward et al., 2000; Suzuki et al., 2003; Ward and Sanders, 2006; Sanders et al., 2014). ICC also play a role in afferent neural signaling and act as non-neuronal stretch receptors (Thuneberg and Peters, 2001; Won et al., 2005; Garcia-Lopez et al., 2009). Kit expression has provided an efficient means of identifying ICC at the light microscopic level (Sanders et al., 1999). ICC are classified into several subtypes, with the lower oesophageal sphincter (LOS) containing only intramuscular ICC (ICC-IM), and ICC at the MP are absent since there is no pacemaker activity in this region. The main role of the ICC-IM subtype is neurotransmission and stretch sensing (Komuro, 2006; Radenkovic et al., 2010a; Radenkovic, 2012).

Loss or dysfunction of ICC in various dysmotilities and in animal models has been shown to lead to gastric dysrhythmias, gastroparesis, slow intestinal transit, impaired neuroeffector mechanisms and altered visceral afferent signaling, which are considered the hallmarks of diabetic gastroenteropathy (Ordog, 2008). Changes in the enteric nervous system and smooth muscle cells were also reported in diabetic patients (Pasricha et al., 2008) and an ICC loss was documented in the stomach of diabetic patients with gastroparesis (He et al., 2001; Forster et al., 2005; Iwasaki et al., 2006; Grover et al., 2011; Kim et al., 2012). Animal model studies with type I and type II diabetes models also showed a loss of ICC. In the studies with non-obese diabetic (NOD) mice, a model of human type I diabetes, ICC network reduction has been noted in gastric corpus and antrum (Ordog et al., 2000; Horvath et al., 2006). In the type II diabetes mice model (leptin receptor mutant mice), a modest ICC count decline appeared in both MP and ICC-IM throughout the stomach, small intestine and colon (Yamamoto et al., 2008). The loss of ICC was also detected in the gastric fundus and corpus of streptozotocin (STZ)-diabetic rats, along with a depletion of ICC-IM in both circular and longitudinal muscle layers (Wang et al., 2009). A more suitable diabetes animal model is the animal model created by Masiello (Masiello et al., 1998), inducing diabetes by the administration of two compounds: STZ and nicotinamide (NA). A recent review shows the versatility of the STZ-NA animal model (Szkudelski, 2012). The severity of STZ–NA-induced diabetes is much lower than that of diabetes induced by STZ alone; rats manifest moderate hyperglycemia and do not require exogenous insulin to survive due to preserved insulin-secreting response to glucose, and this also represents an appropriate model for human type II diabetes. We have not found any data about ICC and its distribution and changes in the STZ-NA rat model.

Blueberries are known to possess anti-diabetic activity (Basu et al., 2010; Stull et al., 2010; Babu et al., 2013; Chang et al., 2013), and have been used in traditional medicine for a variety of conditions. Bilberries (*Vaccinium myrtillus*) particularly, are one of the richest sources of antioxidants (Takikawa et al., 2010). To date, there have been numerous studies that examine and demonstrate antioxidant, protective, hypoglycemic effects of bilberries in diabetes (Yang et al., 2005; Grace et al., 2009; Takikawa et al., 2010; Asgary et al., 2015); however there is no data on the effect of bilberries and possible protective effects on the ICC in diabetes.

The objective of the present study was to investigate the alterations of ICC in the LOS of STZ-NA noninsulin-dependent diabetes rats. We also investigated the possible changes in ICC distribution in rats with the same type of diabetes and treated with the bilberry fruit extract.

Materials and methods

Animals

Male Wistar rats (weighing 230-250 g and 10 weeks old) were used in the present study. The study was carried out in the Research Center for Biomedicine (Faculty of Medicine, University of Nis, Nis, Serbia) in a controlled environment. Animals were placed into four groups (seven animals per group): diabetic (STZ-NA rats), age-matched controls, diabetic animals treated with bilberry and controls treated with the same dosage of bilberry extract. All groups had unlimited access to food and water during the experiment. Non-insulin dependent diabetes was induced by an intraperitoneal injection of streptozotocin (Sigma Aldrich, SAD) at a dosage of 45 mg/kg body weight immediately after intraperitoneal application of nicotinamide (Sigma Aldrich, SAD) at a dosage of 110 mg/kg body weight. Hyperglycemia was verified using the Accu-check Performa (Roche) glucose meter on 3rd and 7th day after the administration of STZ and NA. Blood samples were collected from the tail vein after 2 hours' fasting. Animals with a glucose level of >8.3 mmol/L were considered diabetic. After four weeks, with the animal diabetic model fully developed, the oral treatment with bilberry fruit extract (10.6 mg/ml of anthocyanins equivalents) was started for two weeks at a dose of 50 mg/kg body weight. After two weeks, the animals were sacrificed by exsanguination after bilateral thoracotomy in deep anesthesia (ketamine hydrochloride, 100 mg/kg body weight). Animals were kept in accordance with the National Guide for the Care and Use of Laboratory Animals, and all experimental procedures had been previously approved by the Ethics Committee of the Faculty of Medicine, University of Nis.

Bilberry source and preparation of the fruit extract

Bilberries (*Vaccinium myrtillus* L.) were sampled at the full ripe stage in the woods from Koroška and Škofja Loka, Slovenia and stored at -20°C for one week when methanol extracts were prepared. The extraction method was modified and improved in accordance with an already reported method (Moze et al., 2011). Methanol was evaporated using a rotary vacuum evaporator at low temperature, and the concentration of anthocyanin equivalents was calculated to 10.6 mg/ml.

Tissue preparation

The stomach with lower oesophagus was removed from rats via abdominal incision immediately after the exsanguination, and immersed in a saline solution. The stomach was opened along the greater curvature and gastric contents were washed with saline solution. Cardia and the lower esophagus were isolated and fixed in formaldehyde (10%).

Immunohistochemistry

The specimens were exposed to anti-c-kit antibodies to investigate ICC, and the smooth muscle cells were immunohistochemically labelled with anti-desmin (DES) antibodies. The sections were deparaffined in xylol and a descending series of alcohol rinses (less than 1 min each) followed by rehydration in distilled water. Blocking of endogenous peroxydase was performed with 3% H_2O_2 for 10 min. After that, incubation with primary antibodies was performed for 60 min at room temperature, followed by rinses in a phosphate-buffered solution (0.1 M PBS, pH 7.4). The primary antibodies were diluted in Dako antibody diluent (catalogue no. S0809; Dako North America, Carpinteria, CA, USA). Incubation was performed with streptavidin horseradish conjugate for 30 min at room temperature, and DAKO Liquid DAB + Substrate/Chromogen System (code no. K3468) was used for complex visualization. Mayer's haematoxylin was used for counterstaining of all immunolabelled sections. Immunoreactivity was absent in negative controls in which the primary antibody was omitted. The primary antibodies used and their respective dilutions are listed in Table 1.

Quantitative image analysis

Circular and longitudinal muscle area and numerical areal density (N_A) of ICC were determined by digital image analysis using Image J software (National Institutes of health, Maryland, USA, http://imagej.nih.gov/ij/). The images of the muscle layer were obtained on Olympus BX 50 light microscope equipped with a digital camera (Leica Micro-Systems, Reuil-Malmaison, France), by systematic random sampling method. The objective ×40 was applied to determine the numerical areal density of ICC, i.e. the average number of cells per mm^2 of circular or longitudinal muscle tissue. The cells were counted manually in order to avoid c-kit positive mast cells, which differ from ICC in shape, granular content and localization. The obtained values for numerical areal density were compared between the groups by using the Kruskal-Wallis One Way Analysis of Variance on Ranks.

Results

Blood glucose levels after two hours' fasting were significantly higher ($p<0.001$) in STZ-NA rats (10.81±0.57 mmol/l) compared to controls (6.53±0.69 mmol/l). Bilberry extract had a lowering effect on blood glucose values in STZ-NA animals (10.81±0.57 mmol/l vs. 9.88±0.21 mmol/l, $p<0.001$; controls: 6.53±0.69 mmol/l vs. 6.56±0.57 mmol/l). The STZ-NA treated rats did not present significant changes in body weight; however, they had moderate polydipsia and polyfagia. During the sacrifice procedure, distension of the intestines was observed in diabetic animals, and a lower degree of distension in diabetic rats treated with bilberries. Diabetic animals on bilberry treatment had moderate, although not significant polydipsia. Polyfagia was not observed in those animals. There was no significant difference in body weight among groups at the end of the experiment.

In control rat LOS (Fig. 1A), c-kit-IR cells were present in large numbers within both muscle layers. DES immunoreactivity was present in the circular and longitudinal muscle layers (Fig. 1B). C-kit-IR cells identifiable as ICC-IM were more numerous in the circular layer than in the longitudinal one (Fig. 1C). These cells were spindle-shaped, with two long, thin processes originating from the opposite poles. They ran parallel to the longitudinal axis of the smooth muscle cells and (based on topographical and morphological criteria) they corresponded to the ICC-IM (Fig. 1C,D). ICC were not present either at the submucosal border of the circular muscle layer, or between the two muscle layers (Fig. 3). Most commonly they were single, but long linear cell-cell connections were also seen (Fig. 2A,B). Very often, ICC formed long rows of interconnected cells within the circular muscle layer (Fig. 2 B).

In STZ-NA rat LOS, there were no signs of necrosis or apoptosis and there was no evidence of neutrophil or

Table 1. Antibodies.

Antigen	Clone	Supplier	Dilution
C-kit	CD-117	Dako	1:100
Desmin	DE-R-11	Dako	1:100

lymphocyte infiltration. ICC were present in both muscule layers, but the density of these cells was significantly reduced (Fig. 2D). In the LOS of diabetic rats, ICC-IM were rarely present and linear cell-cell connections between these cells were completely missing. ICC-IM were present as a single cell in the visual field, and in some of the samples the so-called "empty fields" were also observed, with a complete lack of c-kit-IR cells (Fig. 2D). The results for numerical areal density (N_A) of ICC in circular and longitudinal muscle of the experimental animals show that there is a statistically significant difference (P=<0.001) in the number of ICC between the control and STZ-NA group (Fig. 3.)

In the group treated with bilberry, ICC were present in both muscle layers of the LOS (Fig. 2E). Their number and distribution were exactly the same as in the rats with diabetes who were not treated with bilberry, there is not a statistically significant difference in NA of ICC in circular and longitudinal muscle between STZ-NA group and group treated with bilberry. However, there was a difference in the N_A of ICC in circular and longitudinal muscle between control and diabetic group with bilberry treatment, as shown in Fig. 3.

In addition to ICC, we also found a large number of c-kit-IR mast cells (Fig. 2C) in all experimental groups. However, their shape and granular content, as well as their localization, enabled us to distinguish them from ICC. Mast cells were present especially within the lamina propria between the glands, in the submucosa and within the connective tissue septa of the muscle layers (Fig. 2C).

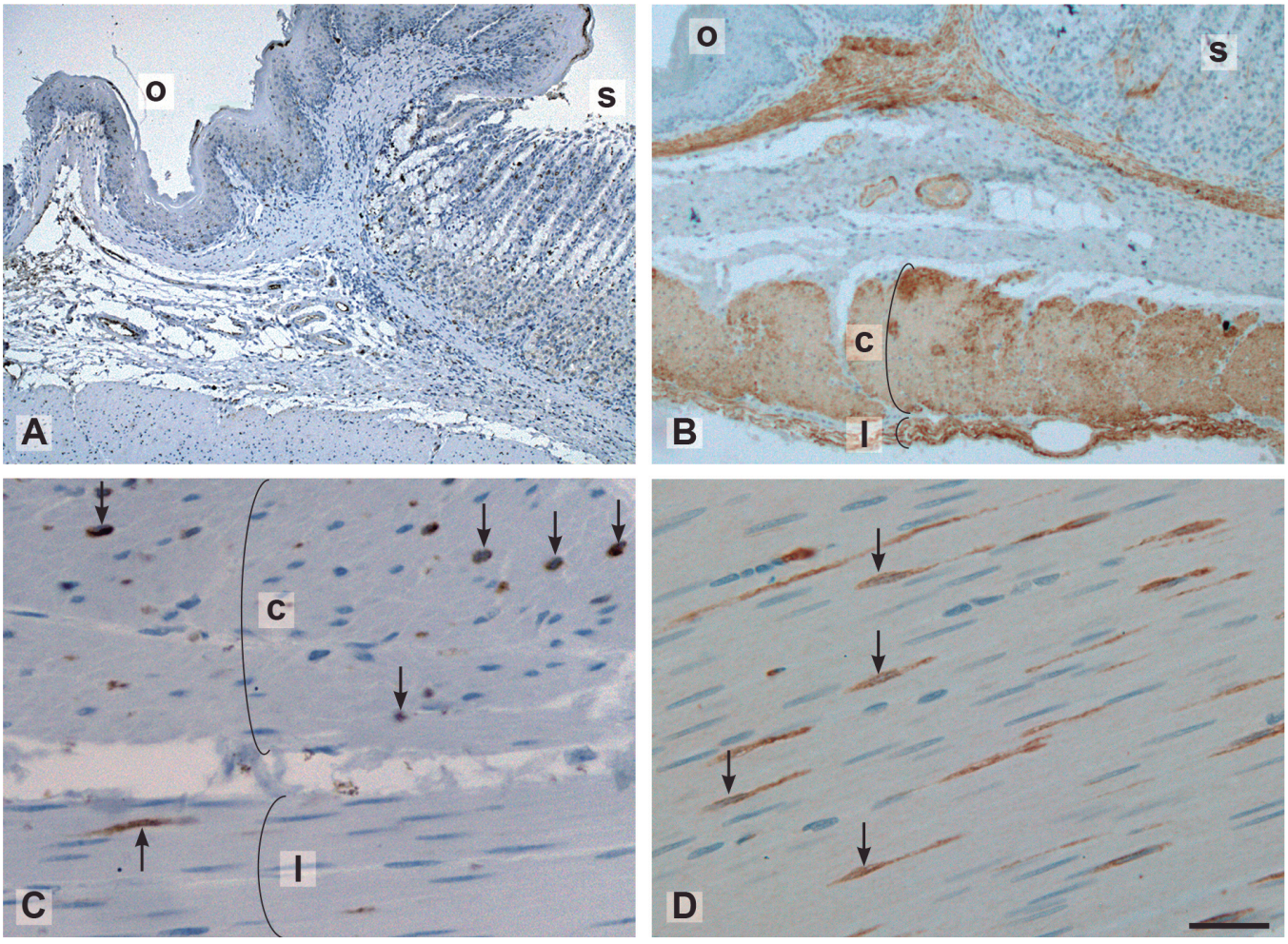

Fig. 1. c-kit **(A, C and D)** and desmin **(B)** immunohistochemistry. **A.** The cardias (s) and the lower oesophagus (o) of control group of rats. **B.** Desmin-IR was present in the wide circular (c) and the thin longitudinal (l) muscle layers of LOS. **C.** The circular (c) and longitudinal muscle layers of LOS of control group. c-kit IR cells (arrows) identifiable as ICC-IM, were distributed parallel to the longitudinal axis of the smooth muscle cells within both muscle layers. **D.** The circular muscle layer of LOS of control group. C-kit IR cells (arrows) were numerous. They had two long processes originating from their opposite poles. Scale bars: A, 200 μm; B, 100 μm; C, D, 20 μm.

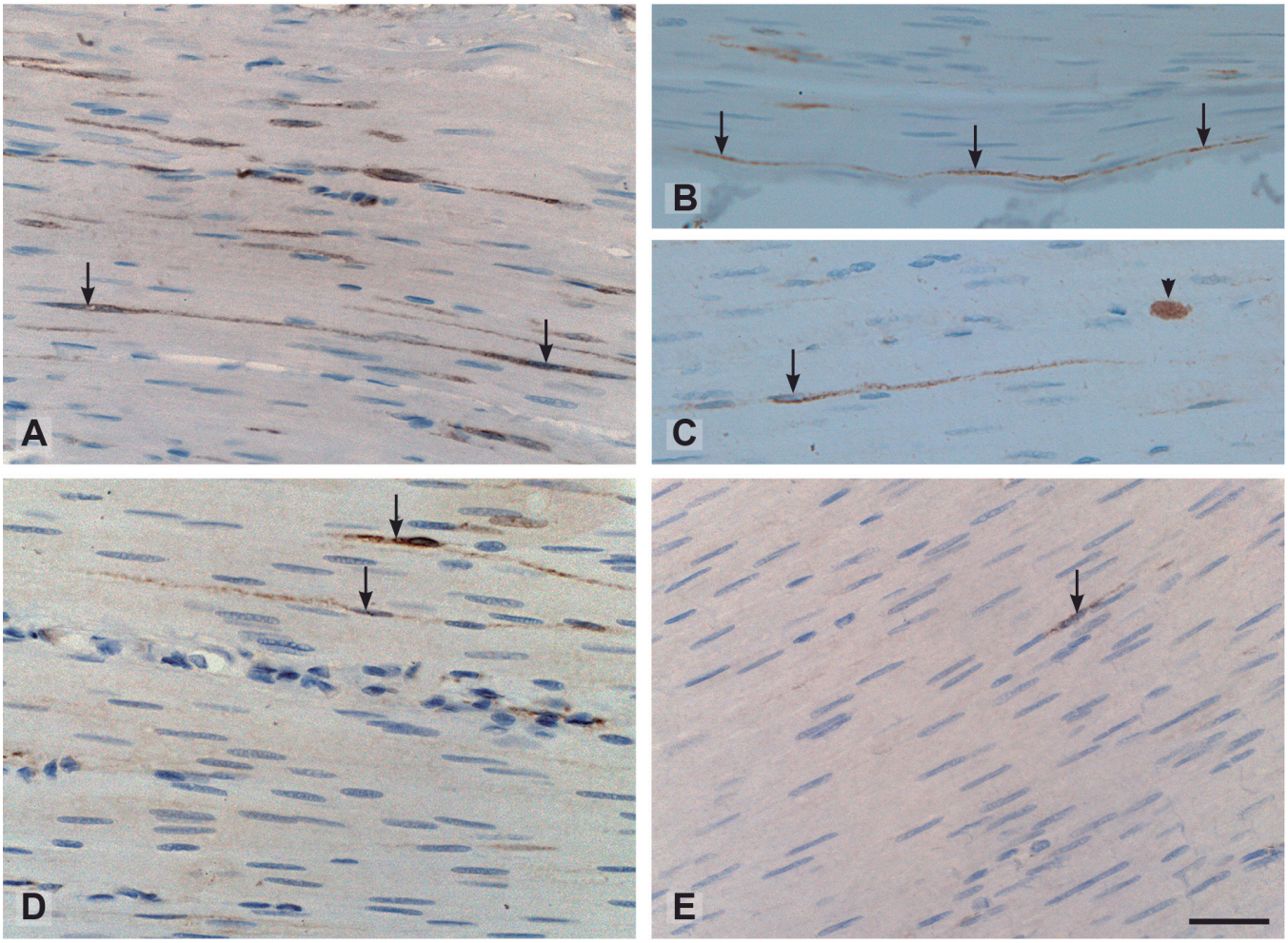

Fig. 2. c-kit immunohistochemistry. **A.** The circular muscle layer of LOS of control group. Most commonly c-kit IR ICC-IM were single, but also long linear cell-cell connections were seen (arrows). **B.** Three ICC were interconnected (arrows) and oriented parallel to the long axis of the smooth muscle cells. **C.** An intramuscular ICC (arrow) with a very long and thin processus. An isolated oval c-kit-IR mast cell (arrow-head) was located within the intramuscular connective-tissue septa. **D.** The circular muscle layer of LOS of STZ-NA rats. The ICC (arrows) were rarer compared with the control group. **E.** The circular muscle layer of LOS of STZ-NA rats treated with bilberry. Spindle-shaped ICC (arrow) were few as in the STZ-NA rats. They ran parallel to the longitudinal axis of the smooth muscle cells. Scale bars: 20 μm.

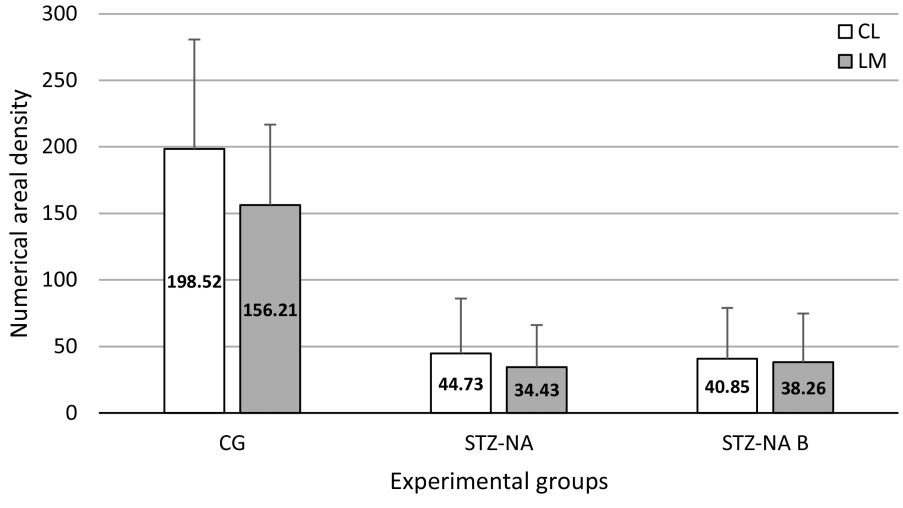

Fig. 3. Numerical areal density of interstitial cells of Cajal in the circular and longitudinal muscle in examined experimental groups. CG-control group; STZ-NA: diabetes induced group; STZ-NA B: diabetes group treated with bilberry; CM: circular muscle; LM: longitudinal muscle.

Discussion

In the STZ-NA animal model, diabetes is induced by the administration of two compounds: STZ and NA. STZ is a well-known diabetogenic agent exerting cytotoxic action on pancreatic B-cells, whereas NA is given to rats to partially protect these cells against STZ. The advantages of this model, compared to STZ alone model and previous animal models that examined ICC loss in diabetes, are that this STZ-NA induced diabetes animal model is more similar to human type II diabetes; rats manifest moderate hyperglycemia and do not require exogenous insulin to survive. Unlike other STZ studies (Wang et al., 2009), STZ-NA treated rats did not have significant changes in body weight.

C-kit-positive ICC-IM were found to be densely distributed throughout the circular and longitudinal muscle layers of the LOS, in all animal groups. However, ICC were not found within the myenteric and submucous regions. This ICC distribution is similar to that seen in the LOS of the human foetus from the fourth month of development (Radenkovic et al., 2010b) and mouse (Komuro, 2006).

There was a decreased number of ICC in STZ-NA rat LOS. ICC depletion in diabetes could have potentially arisen from hyperglycaemia and associated oxidative damage, reduction of insulin and growth factor signaling, which caused dystrophic cell changes, autoimmune attack, or their combinations.

Increased oxidative stress, associated with hyperglycaemia has been reported in diabetic NOD mice with gastroparesis along with reduced expression of neuronal NO synthase and heme oxygenase-1, which are potentially cytoprotective and a survival factor for ICC (Choi et al., 2007). The loss of ICC and associated nerve fibers suggests the possibility of interdependence, as proposed in the studies of ICC-IM and vagal afferent nerves in animal models (Huizinga et al., 2008; Powley et al., 2008), and in patients (Iwasaki et al., 2006). Wang at al. observed ICC loss in diabetic STZ rats, and also nerve fibers, but not nerve cell bodies. Nerve fiber loss was due to the ICC-IM loss and partial depletion of synapse-like connections with ICC-IM (Wang et al., 2009). These findings emphasize the importance of ICC, and also show how ICC alterations occurring in diabetes, could be one of the major factors in the development of gastroenteric neuropathy. In addition, in the present study we demonstrated an ICC loss in moderate hyperglicaemia diabetes. Some recent studies investigating ICC loss in NOD mice (model of human type 1 diabetes), have shown that the main cause of ICC loss is not hyperglycemia, but reduced insulin-like growth factor-1 (IGF-I) and insulin signaling in diabetes (Horvath et al., 2005). Ordog et al. observed a reduced ICC number in the antrum and loss of ICC nerve junctions with motor neurons in the fundus of NOD mice (Ordog et al., 2000), which was hypothesized to be due to reduced insulin and IGF-I signaling (Horvath et al., 2006), and which might have been relevant in our model as well. Insulin and IGF-I completely prevented the loss of ICC in the murine gastric *tunica muscularis* cell cultures (Horvath et al., 2005), and loss of ICC and nerve structures due to absence of growth factors was consistent with metabolic injury in diabetes. Diabetic gastroneuropathy functional defects, such as low muscle response to enteric motor neuron activation, reduced and arrhythmic gastric slow wave activity and delayed gastric emptying were associated with ICC changes (Ordog et al., 2000; Choi et al., 2008).

However, the antidiabetic effect of blueberries, besides that of lowering blood glucose levels, is to reduce apoptosis by 20-33% in the cells exposed to elevated glucose values and to reduce the level of oxidative stress (Martineau et al., 2006). Researches have shown that blueberries contain some active principles with insulin-like and glitazone-like properties, with a protective effect against toxicity induced by elevated glucose values (Martineau et al., 2006). It was to be expected that bilberry has got a protective effect on ICC. However, in the present study, the distribution and number of ICC completely coincided with the ICC described in diabetes group untreated with bilberry. Our results showed that bilberry had no effect on ICC. Moreover, there is no data in the literature about whether and how blueberries can affect ICC in the digestive tract.

In both patients and animal models, cell loss appears to be the main cause of ICC-related pathologies. Farre et al. demonstrated that the lack of ICC was related to a basal tone increase and spontaneous contractile activity in the rat LOS (Farre et al., 2007). In the present study, we did not find linear cell-cell ICC-IM connections in STZ-NA as in rat control group. A loss of ICC-IM cells together with loss of connectivity between ICC-IM and ICC-neuronal network may interfere with the relaxation and normal motility in this sphincter with ectopic and multiple pacemaker sites, which could lead to various patterns of electrical dysrhythmia. An injury to ICC-IM may be responsible for gastric dysrhythmias in patients (Forster et al., 2005) and STZ rat model (Wang et al., 2009), and can be applied to this study as well, analyzing the effects of moderate hyperglycaemia.

Immune cells may be involved in the loss and degenerative changes of ICC. In our study there was no conspicuous evidence of an increase in immune cells (including lymphocytes, macrophages or neutrophils) in the LOS muscle coat of STZ-NA rats, compared with control. Mast cells were seen in an increased number within the stomach wall of both experimental groups with diabetes (STZ-NA and STZ-NA with bilberry treatment). In human studies of Crohn's disease and achalasia, a close contact between injured ICC and mast cells was found, where the hypothesis was that mast cells were involved in ICC maintenance and repair in response to growth factor secretion (Zarate et al., 2006; Wang et al., 2007). However, although we noticed an increased number of mast cells, the density of ICC was significantly reduced in both groups with diabetes.

One of the causes of gastroenteropathy may be an

autoimmune attack. Loss of ICC caused by anti-Kit autoantibodies has been reported in a patient with paraneoplastic gastroparesis and intestinal pseudo-obstruction (Pardi et al., 2002). Nevertheless, in diabetes, an autoimmune attack on ICC has not been demonstrated (Ordog, 2008).

In addition, it is impossible to completely exclude the stress and stressogenic environmental factors. A STZ-NA rat may be under stress as a contributing factor, although such a stress factor should be similar for both control and STZ-NA animals.

A reduction of ICC-IM number and degenerative changes in the residual ICC have been previously described in the gastric fundus and corpus of STZ rats (Wang et al., 2009). Although in previous studies no ICC loss was found in longterm diabetic NOD mice but the typical close relationships between ICC and enteric nerve terminals were absent due to extracellular matrix accumulation (Ordog et al., 2000), our results showed that there was a significant reduction in the number of ICC-IM and disruption of linear arrays of these cells, which indicated that neurotransmission was affected. In earlier studies using mutant Ws/Ws rats and W/Wv mice, which lacked intramuscular ICC in the stomach, it was shown that there was an impaired muscle basal tone of the entire proximal stomach, LOS and cardias (Sivarao et al., 2001; Dixit et al., 2006; Farre et al., 2007). The explanation for the reduced tone and impaired nitrergic relaxation in diabetes may lie in the ICC involvement.

In summary, a decrease of ICC-IM number, discontinuities and breakdown of contacts between them were observed in STZ-NA induced non-insulin-dependent diabetes and in the group treated with bilberry. Based on that, we can conclude that impared LOS relaxation and motility could be expected in diabetic gastroenteropathy. Bilberry fruit extract was shown to have hypoglycemic activity, but there was no protective effect on ICC in the LOS of diabetic rats.

Acknowledgements. This study was supported by the project III43012 funded by Ministry of Education and Science of Republic Serbia

References

Asgary S., RafieianKopaei M., Sahebkar A., Shamsi F. and Goli-Malekabadi N. (2015). Anti-hyperglycemic and anti-hyperlipidemic effects of vaccinium myrtillus fruit in experimentally-induced diabetes (antidiabetic effect of vaccinium myrtillus fruit). J. Sci. Food Agric. 96, 764-768.

Babu P.V.A., Liu D. and Gilbert E.R. (2013). Recent advances in understanding the anti-diabetic actions of dietary flavonoids. J. Nutr. Biochem. 24, 1777-1789.

Bagyanszki M. and Bodi N. (2012). Diabetes-related alterations in the enteric nervous system and its microenvironment. World J. Diabetes 3, 80-93.

Basu A., Du M., Leyva M.J., Sanchez K., Betts N.M., Wu M., Aston C.E. and Lyons T.J. (2010). Blueberries decrease cardiovascular risk factors in obese men and women with metabolic syndrome. J. Nutr. 140, 1582-1587.

Beckett E.A., Ro S., Bayguinov Y., Sanders K.M. and Ward S.M. (2007). Kit signaling is essential for development and maintenance of interstitial cells of cajal and electrical rhythmicity in the embryonic gastrointestinal tract. Dev. Dyn. 236, 60-72.

Boulton A.J., Vinik A.I., Arezzo J.C., Bril V., Feldman E.L., Freeman R., Malik R.A., Maser R.E., Sosenko J.M. and Ziegler D. (2005). Diabetic neuropathies: A statement by the american diabetes association. Diabetes Care 28, 956-962.

Chang C.L., Lin Y., Bartolome A.P., Chen Y.C., Chiu S.C. and Yang W.C. (2013). Herbal therapies for type 2 diabetes mellitus: Chemistry, biology, and potential application of selected plants and compounds. Evidence-based complementary and alternative medicine: eCAM 2013, 378657.

Choi K.M., Gibbons S.J., Roeder J.L., Lurken M.S., Zhu J., Wouters M.M., Miller S.M., Szurszewski J.H. and Farrugia G. (2007). Regulation of interstitial cells of cajal in the mouse gastric body by neuronal nitric oxide. Neurogastroenterol. Motil. 19, 585-595.

Choi K.M., Gibbons S.J., Nguyen T.V., Stoltz G.J., Lurken M.S., Ordog T., Szurszewski J.H. and Farrugia G. (2008). Heme oxygenase-1 protects interstitial cells of cajal from oxidative stress and reverses diabetic gastroparesis. Gastroenterology 135, 2055-2064.

Dixit D., Zarate N., Liu L.W., Boreham D.R. and Huizinga J.D. (2006). Interstitial cells of cajal and adaptive relaxation in the mouse stomach. Am. J. Physiol. Gastrointest. Liver Physiol. 291, G1129-1136.

Farre R., Wang X.Y., Vidal E., Domenech A., Pumarola M., Clave P., Huizinga J.D. and Jimenez M. (2007). Interstitial cells of cajal and neuromuscular transmission in the rat lower oesophageal sphincter. Neurogastroenterol. Motil. 19, 484-496.

Forster J., Damjanov I., Lin Z., Sarosiek I., Wetzel P. and McCallum R.W. (2005). Absence of the interstitial cells of cajal in patients with gastroparesis and correlation with clinical findings. J. Gastrointest. Surg. 9, 102-108.

Garcia-Lopez P., Garcia-Marin V., Martinez-Murillo R. and Freire M. (2009). Updating old ideas and recent advances regarding the interstitial cells of cajal. Brain Res. Rev. 61, 154-169.

Grace M.H., Ribnicky D.M., Kuhn P., Poulev A., Logendra S., Yousef G.G., Raskin I. and Lila M.A. (2009). Hypoglycemic activity of a novel anthocyanin-rich formulation from lowbush blueberry, vaccinium angustifolium aiton. Phytomedicine 16, 406-415.

Grover M., Farrugia G., Lurken M.S., Bernard C.E., Faussone-Pellegrini M.S., Smyrk T.C., Parkman H.P., Abell T.L., Snape W.J., Unalp-Arida A., Nguyen L., Koch K.L., Calles J., Lee L., Tonascia J., Hamilton F.A. and Pasricha P.J. (2011). Cellular changes in diabetic and idiopathic gastroparesis. Gastroenterology 140, 1575-1585.

He C.L., Soffer E.E., Ferris C.D., Walsh R.M., Szurszewski J.H. and Farrugia G. (2001). Loss of interstitial cells of cajal and inhibitory innervation in insulin-dependent diabetes. Gastroenterology 121, 427-434.

Horvath V.J., Vittal H. and Ordog T. (2005). Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of cajal in the murine stomach. Diabetes 54, 1528-1533.

Horvath V.J., Vittal H., Lorincz A., Chen H., Almeida-Porada G., Redelman D. and Ordog T. (2006). Reduced stem cell factor links smooth myopathy and loss of interstitial cells of cajal in murine diabetic gastroparesis. Gastroenterology 130, 759-770.

Huizinga J.D. and Lammers W.J. (2009). Gut peristalsis is governed by a multitude of cooperating mechanisms. Am. J. Physiol. Gastrointest. Liver Physiol. 296, G1-8.

Huizinga J.D., Reed D.E., Berezin I., Wang X.Y., Valdez D.T., Liu L.W. and Diamant N.E. (2008). Survival dependency of intramuscular icc on vagal afferent nerves in the cat esophagus. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R302-310.

Hwang S.J., Blair P.J., Britton F.C., O'Driscoll K.E., Hennig G., Bayguinov Y.R., Rock J.R., Harfe B.D., Sanders K.M. and Ward S.M. (2009). Expression of anoctamin 1/tmem16a by interstitial cells of cajal is fundamental for slow wave activity in gastrointestinal muscles. J. Physiol. 587, 4887-4904.

Iwasaki H., Kajimura M., Osawa S., Kanaoka S., Furuta T., Ikuma M. and Hishida A. (2006). A deficiency of gastric interstitial cells of cajal accompanied by decreased expression of neuronal nitric oxide synthase and substance p in patients with type 2 diabetes mellitus. J. Gastroenterol. 41, 1076-1087.

Kim E.R., Kim K.M., Lee J.Y., Joo M., Kim S., Noh J.H., Ward S.M., Koh S.D. and Rhee P.L. (2012). The clue of interstitial cell of cajalopathy (iccpathy) in human diabetic gastropathy: The ultrastructural and electrical clues of iccpathy in human diabetic gastropathy. Exp. Toxicol. Pathol. 64, 521-526.

Komuro T. (2006). Structure and organization of interstitial cells of cajal in the gastrointestinal tract. J. Physiol. 576, 653-658.

Martineau L.C., Couture A., Spoor D., Benhaddou-Andaloussi A., Harris C., Meddah B., Leduc C., Burt A., Vuong T., Mai Le P., Prentki M., Bennett S.A., Arnason J.T. and Haddad P.S. (2006). Anti-diabetic properties of the canadian lowbush blueberry *Vaccinium angustifolium* ait. Phytomedicine 13, 612-623.

Masiello P., Broca C., Gross R., Roye M., Manteghetti M., Hillaire-Buys D., Novelli M. and Ribes G. (1998). Experimental niddm: Development of a new model in adult rats administered streptozotocin and nicotinamide. Diabetes 47, 224-229.

Moze S., Polak T., Gasperlin L., Koron D., Vanzo A., Poklar Ulrih N. and Abram V. (2011). Phenolics in slovenian bilberries (*Vaccinium myrtillus* l.) and blueberries (*Vaccinium corymbosum* l.). J. Agric. Food Chem. 59, 6998-7004.

Ordog T. (2008). Interstitial cells of cajal in diabetic gastroenteropathy. Neurogastroenterol. Motil. 20, 8-18.

Ordog T., Takayama I., Cheung W.K., Ward S.M. and Sanders K.M. (2000). Remodeling of networks of interstitial cells of cajal in a murine model of diabetic gastroparesis. Diabetes 49, 1731-1739.

Ordog T., Hayashi Y. and Gibbons S.J. (2009). Cellular pathogenesis of diabetic gastroenteropathy. Minerva Gastroenterol. Dietol. 55, 315-343.

Pardi D.S., Miller S.M., Miller D.L., Burgart L.J., Szurszewski J.H., Lennon V.A. and Farrugia G. (2002). Paraneoplastic dysmotility: Loss of interstitial cells of Cajal. Am. J. Gastroenterol. 97, 1828-1833.

Pasricha P.J., Pehlivanov N.D., Gomez G., Vittal H., Lurken M.S. and Farrugia G. (2008). Changes in the gastric enteric nervous system and muscle: A case report on two patients with diabetic gastroparesis. BMC Gastroenterol. 8, 21.

Powley T.L., Wang X.Y., Fox E.A., Phillips R.J., Liu L.W. and Huizinga J.D. (2008). Ultrastructural evidence for communication between intramuscular vagal mechanoreceptors and interstitial cells of Cajal in the rat fundus. Neurogastroenterol. Motil. 20, 69-79.

Radenkovic G. (2012). Two patterns of development of interstitial cells of Cajal in the human duodenum. J. Cell. Mol. Med. 16, 185-192.

Radenkovic G., Savic V., Mitic D., Grahovac S., Bjelakovic M. and Krstic, M. (2010a). Development of c-kit immunopositive interstitial cells of Cajal in the human stomach. J. Cell. Mol. Med. 14, 1125-1134.

Radenkovic G., Ilic I., Zivanovic D., Vlajkovic S., Petrovic V. and Mitrovic O. (2010b). C-kit-immunopositive interstitial cells of Cajal in human embryonal and fetal oesophagus. Cell Tissue Res. 340, 427-436.

Sanders K.M., Ordog T., Koh S.D., Torihashi S. and Ward S.M. (1999). Development and plasticity of interstitial cells of Cajal. Neurogastroenterol. Motil. 11, 311-338.

Sanders K.M., Salter A.K., Hennig G.W., Koh S.D., Perrino B.A., Ward S.M. and Baker S.A. (2014). Responses to enteric motor neurons in the gastric fundus of mice with reduced intramuscular interstitial cells of Cajal. J. Neurogastroenterol. Motil. 20, 171-184.

Sfarti C., Trifan A., Hutanasu C., Cojocariu C., Singeap A.M. and Stanciu C. (2010). Prevalence of gastroparesis in type 1 diabetes mellitus and its relationship to dyspeptic symptoms. J. Gastrointestin. Liver Dis. 19, 279-284.

Sivarao D.V., Mashimo H.L., Thatte H.S. and Goyal R.K. (2001). Lower esophageal sphincter is achalasic in nnos(-/-) and hypotensive in w/w(v) mutant mice. Gastroenterology 121, 34-42.

Stull A.J., Cash K.C., Johnson W.D., Champagne C.M. and Cefalu W.T. (2010). Bioactives in blueberries improve insulin sensitivity in obese, insulin-resistant men and women. J. Nutr. 140, 1764-1768.

Suzuki H., Ward S.M., Bayguinov Y.R., Edwards F.R. and Hirst G.D. (2003). Involvement of intramuscular interstitial cells in nitrergic inhibition in the mouse gastric antrum. J. Physiol. 546, 751-763.

Szkudelski T. (2012). Streptozotocin-nicotinamide-induced diabetes in the rat. Characteristics of the experimental model. Exp. Biol. Med. 237, 481-490.

Takaki M. (2003). Gut pacemaker cells: The interstitial cells of Cajal (ICC). J. Smooth Muscle Res. 39, 137-161.

Takikawa M., Inoue S., Horio F. and Tsuda T. (2010). Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. J. Nutr. 140, 527-533.

Thuneberg L. and Peters S. (2001). Toward a concept of stretch-coupling in smooth muscle. I. Anatomy of intestinal segmentation and sleeve contractions. Anat. Rec. 262, 110-124.

Torihashi S., Ward S.M., Nishikawa S., Nishi K., Kobayashi S. and Sanders K.M. (1995). C-kit-dependent development of interstitial cells and electrical activity in the murine gastrointestinal tract. Cell Tissue Res. 280, 97-111.

Vinik A.I., Maser R.E., Mitchell B.D. and Freeman R. (2003). Diabetic autonomic neuropathy. Diabetes Care 26, 1553-1579.

Wang X.Y., Huizinga J.D., Diamond J. and Liu L.W. (2009). Loss of intramuscular and submuscular interstitial cells of Cajal and associated enteric nerves is related to decreased gastric emptying in streptozotocin-induced diabetes. Neurogastroenterol. Motil. 21, 1095-e92.

Wang X.Y., Zarate N., Soderholm J.D., Bourgeois J.M., Liu L.W. and Huizinga J.D. (2007). Ultrastructural injury to interstitial cells of Cajal and communication with mast cells in crohn's disease. Neurogastroenterol. Motil. 19, 349-364.

Ward S.M. (2000). Interstitial cells of Cajal in enteric neurotransmission. Gut. 47 (Suppl 4), 40-43.

Ward S.M. and Sanders K.M. (2006). Involvement of intramuscular interstitial cells of cajal in neuroeffector transmission in the gastrointestinal tract. J. Physiol. 576, 675-682.

Ward S.M., Beckett E.A., Wang X., Baker F., Khoyi M. and Sanders K.M. (2000). Interstitial cells of cajal mediate cholinergic neurotransmission from enteric motor neurons. J. Neurosci. 20, 1393-1403.

Won K.J., Sanders K.M. and Ward S.M. (2005). Interstitial cells of Cajal mediate mechanosensitive responses in the stomach. Proc. Natl. Acad. Sci. USA 102, 14913-14918.

Yamamoto T., Watabe K., Nakahara M., Ogiyama H., Kiyohara T., Tsutsui S., Tamura S., Shinomura Y. and Hayashi N. (2008). Disturbed gastrointestinal motility and decreased interstitial cells of cajal in diabetic db/db mice. J. Gastroenterol. Hepatol. 23, 660-667.

Yang Q., Graham T.E., Mody N., Preitner F., Peroni O.D., Zabolotny J.M., Kotani K., Quadro L. and Kahn B.B. (2005). Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436, 356-362.

Zarate N., Wang X.Y., Tougas G., Anvari M., Birch D., Mearin F., Malagelada J.R. and Huizinga J.D. (2006). Intramuscular interstitial cells of Cajal associated with mast cells survive nitrergic nerves in achalasia. Neurogastroenterol. Motil. 18, 556-568.

Accepted October 5, 2016