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RESEARCH ARTICLE

Evaluation of Histopathological and Immunohistochemical Effects of Metformin HCl-Loaded Beads Formulations in Streptozotocin-Nicotinamide Induced Diabetic Rats

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A B S T R A C T

Metformin hydrochloride (MHCl) widely used for the treatment of Type 2 DM. The aim of this study was to investigate the early stage histopathological and immunohistochemical effects of MHCl and MHCl-loaded Alginate (AL) and AL-Chitosan (CS) beads on the pancreas of Streptozotocin (STZ)-Nicotinamide (NA) induced diabetic rats. The rats in the following groups were sacrificed 72 hours after drug administration and their pancreatic tissues were removed. The healthy rats (Control, Group I), diabetic rats (Group II; the fasting blood glucose level higher than 126 mg/dl), diabetic rats treated with pure drugs (Group III), blank beads (Group IV), MHCl-loaded-AL-CS (Group V) and AL (Group VI) beads. The prepared samples of pancreatic tissues were examined by histopathological and immunohistochemical methods. Severity of the atrophic appearance of langerhans islet in Groups II, III and IV was high. The degree of necrotic and degenerative changes of islets of langerhans in the Groups V and VI were reduced compared to the changes in the other groups (Groups II, III and IV; $P<0.05$). As immunohistochemical, the shrunken and distorted islet of langerhans with marked loss immunopositive β cells were observed for Groups II, III and IV. However, there were improvements in the number of immunopositive β cells and the diameter of islet of langerhans of Groups V and VI ($P<0.05$). MHCl-loaded AL-CS and AL beads might be beneficial to reduce the histopathological changes of pancreas and thereby, the effects of diabetes.

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INTRODUCTION

Diabetes mellitus (DM) as a chronic disease, leads to an increased concentration of glucose in the blood, because the pancreas doesn't produce enough insulin/the body is unable to effectively use the insulin. The global prevalence of diabetes was guessed to be 9% among adults (>18 years) in 2014. And also, it is estimated that 1.5 million people from especially low and middle income countries died directly from diabetes, and also, the number of people with DM would have ascended to 552 million include 438 million people with Type 2 diabetes by the year 2030. WHO reported that in 2030, diabetes will be the 7th leading cause of the death (Shaw *et al.*, 2010; Whiting *et al.*, 2011). There are four types of diabetes mellitus according to the new classification system. These: Type 1 diabetes ("due to β-cell destruction, usually leading to absolute insulin deficiency"); Type 2 diabetes ("due to a progressive insulin secretary defect on the background of insulin resistance"), gestational diabetes, and other specific types due to other causes such as monogenic diabetes syndromes, drug or chemical induced diabetes and diseases of the exocrine pancreas (American Diabetes Association, 2015).

Type 2 DM, which comprises of 90% of diabetic patient all around the world, is also called as non insulin dependent/late or adult-onset. It is characterized by insulin resistance in liver, muscle and adipose tissue, abnormalities, imbalances affecting multiple organ systems (Thulé, 2012; American Diabetes Association, 2015). Due to physical inactivity, unhealthy diet and

obesity, Type 2 DM in adolescent and children has been appeared since the early of 1990s (Goodman, 2013). The patients with Type 2 DM have normal/elevated insulin levels, but, the insulin is ineffective due to the resistant cells and insulin secretion is impaired and there is a decline in beta cell function to secrete insulin when needed. As the diagnosis of Type 2 diabetes is usually delayed, the formation of microvascular and/or macrovascular complications are observed during the diagnosis (Goodman, 2013).

In the treatment of Type 2 DM, regular physical activity, healthy diet and weight loss are suggested as well as pharmacological treatment.

MHCl, a biguanide derivative widely used for the treatment of Type 2 DM, is prescribed approximately to 120 million people worldwide. MHCl is the active compound of the French Lilac (*Galega officinalis*) and recommended for the first line therapy for Type 2 DM (Viollet *et al.*, 2012). As an antihyperglycemic agent, its major effect is to decrease hepatic glucose output. And also, it decreases the glucose absorption in the small intestine, increases insulin-mediated glucose utilization in peripheral tissues and lowers the plasma free fatty acid concentrations (thus reducing substrate availability for gluconeogenesis) (Viollet *et al.*, 2012; McCulloch, 2015). When it decreases the blood glucose concentrations of patients with Type 2 DM, it does not induce overt hypoglycaemia (Viollet *et al.*, 2012). Furthermore, its positive effects on the tyrosine kinase activity and the insulin receptor expression lead to improvement in insulin sensitivity. Metformin increases the plasma level of glucagon-like peptide 1 and stimulates the function of GLP-1 on β-cells, thereby increases insulin secretion and also stimulates incretin receptor gene expression in islets cells via peroxisome proliferator-activated receptor PPAR-α (Grzybowska, 2011; Viollet *et al.*, 2012; Gregg *et al.*, 2014). In another study, treatment with metformin was shown to induce the regeneration of pancreatic β cells and the positive signalling for insulin (Ismail *et al.*, 2015).

The purpose of the study was to evaluate the effects of metformin HCl and metformin HCl-loaded AL and AL-CS beads on the immunohistochemical and morphometric alterations to islets of langerhans beta-cells (pancreatic beta-cells).

MATERIALS AND METHODS

Monoclonal anti-insulin antibody was obtained from Sigma (USA), LSAB®2 System-HRP for use on rat specimens was purchased from DAKO (USA), all other chemicals/reagents used were of analytical grade.

Experimental procedures: The experimental procedure was performed in two parts. While the first part of the study consisted of the preparation of beads formulations, the induction of diabetes in rats and measuring of the fasting glucose levels after drug administration (Mokhtare, 2015), Briefly, 43 male Sprague-Dawley rats (180-250 g) were fed a normal diet and water (under a 12 h light/dark cycle at 24±2°C). Diabetes was induced in rats, fasted overnight, by a single intraperitoneal injection of freshly prepared STZ (40 mg/kg body weight (b.w.)) in citrate buffer (cold; pH 4.5) and NA solution (120 mg/kg b.w.; 15 minutes later). The healthy rats were used as control (Group I; n=6). Diabetic rats with fasting blood glucose levels of higher than 126 mg/dL were divided into five different groups: Group II: diabetic rats (n=8); Group III: diabetic rats treated with MHCl (n=6; 100 mg/kg b.w.); Group IV: diabetic rats treated with blank beads formulation (n=8); Group V: diabetic rats treated with MHCl-loaded AL-CS beads (n=9; equivalent to MHCl 100 mg/kg b.w); Group VI: Diabetic rats treated with MHCl-loaded AL beads (n=6; equivalent to MHCl 100 mg/kg b.w.). The pure drug and the formulations of blank and both MHCl-loaded beads were administered orally to the rats, fasted overnight, and allowed free access to water (Mokhtare, 2015).

The second part (the current study), comprised the investigation the histopathological and immuno-histochemical effects of pure drug and beads formulations on pancreas removed from the rats, was approved by the Ethics Committee of Ataturk University (December 14, 2013-No: 145). Therefore, the rats were sacrificed 72 hours after drug administration under sevoflurane anesthesia and the pancreas of rats in all groups were removed.

Histopathological study: The pancreatic tissue samples were fixed in 10% neutral buffered formalin and embedded into paraffin blocks and sectioned and stained with Hematoxylin and Eosin. The samples were examined under the microscope and scored as normal (-), light (+), mild (++), and severe (+++) according to the atrophic appearance of islet.

Immunohistochemical study: The immunohistochemical procedure were performed using a streptavidin-biotin detection technique (LSAB®2 System-HRP for use on rat specimens). Deparaffinised pancreatic tissue sections were treated with H_2O_2 (3%) for 10 min to inactivate the endogen peroxidases to determine the insulin immunopositive β cells in the pancreatic islet. These sections were heated in the antigen retrieval solution for 10 min in a microwave oven and incubated with monoclonal anti-insulin primary antibody (1/1000) at 37°C for 30 min after washing with phosphate buffered saline (PBS). Then, the sections were washed with PBS and incubated with the biotin-conjugated secondary antibody for 15 min followed by horseradish peroxidase-labeled streptavidin for further 15 min. The slides were treated with diaminobenzidine (DAB) for about 2 min to visualize, then, washed with pure water and counterstained with hematoxylin. The slides were examined under the microscope.

The number of immunopositive β cells and the diameter of islet of langerhans in randomly selected ten different microscopic fields were determined and the numerical values per animal were calculates as follows:

$$\text{Mean value (diameter/}\mu\text{)} = \frac{\text{The total diameter of islet}}{\text{The total number of islet}}$$

$$\text{Mean value (the number of the cells)} = \frac{\text{The total number of the cells in islet}}{\text{The total number of islet}}$$

Statistical analysis: The differences between the groups analyzed using Kruskal Wallis test and Mann Whitney U test for the histopathological results and also One-way

ANOVA analysis with post-hoc Tukey's HSD for immunohistochemical results with SPSS 20.0 (SPSS, Inc., Chicago, IL, USA) (the results were expressed as the mean±standard deviation). P<0.05 indicates a statistically significant difference.

RESULTS

In this study, the early stage histopathological and immunohistochemical effects of MHCl and MHCl-loaded AL and AL-CS beads on the pancreas of STZ-NA induced Type 2 diabetic rats were investigated.

The removed pancreas of STZ-NA induced diabetic rats and the healthy rats sacrificed 72 hours after administration of pure drug and bead formulations at under sevoflurane anesthesia were examined macroscopically and microscopically. In macroscopic examination, it was found that none of the diabetic and healthy rats had macroscopic lesions in the pancreas.

In histopathological examination, pancreatic sections with Hematoxylin and Eosin of rats in Group I showed normal (-) histological characteristics (Fig. 1a). In STZ-NA induced diabetic rats in Group II, any histopathological structure was not observed in exocrine part of pancreas issue of the diabetic rat. However, there are degenerative and necrotic changes in endocrine part of their pancreas issues (severe (+++) Fig. 1b). In the islets of langerhans of the rats in Group III (administered with pure drug; 100 mg/kg b.w.) and Group IV (administered with blank beads) were determined that atrophic appearance (severe (+++)) associated with the necrotic and degenerative change was maintained (Fig. 1c and Fig. 2a, respectively).

The degree of necrotic and degenerative changes of islets of langerhans in the diabetic rats treated with metformin HCl-loaded AL-CS and AL beads (Groups V and VI, respectively) were reduced (mild (++)) compared to the degree of these changes in islets of the other groups (Groups II, III and IV) (Fig. 2b and Fig. 2c, respectively).

In immunohistochemical study, it was observed that the number of immunopositive β cells and the diameter of islet of langerhans of the control group (Group I) were in the normal levels (Fig. 4a, Table 1). In Group II, the shrunken and distorted islet of langerhans with marked loss immunopositive β cells were observed (Fig. 4b, Table 1). Sections of pancreas of diabetic rats treated with pure drug (Group III) and blank beads (Group IV) showed the similar changes as those in Group II (Fig. 4c and Fig. 5a, respectively). Fig. 5b and Fig. 5c showed that there were improvements in the number of immunopositive β cells and the diameter of islet of langerhans of diabetic rats treated with drug-loaded AL-CS (Group V) and AL beads (Group VI), respectively.

DISCUSSION

DM is a chronic disease requires a life-long treatment process, thus, it has a great impact on the quality of patient's life (Salem and El-Azab, 2014). There were many histopathological and immunohistochemical studies about DM (Aboonabi1 *et al.*, 2014; Abunasef *et al.*, 2014, Rathinam *et al.*, 2014; Szkudelska *et al*., 2014). New treatment approaches are necessary to enhance/improve the efficiency of present antidiabetic agents, to reduce the costs related to the treatment of DM/the management of its complications and to increase the quality of patient's life. Polymeric drug delivery systems (e.g. beads, microspheres, nanospheres) have some advantages such as less variation in the gastrointestinal transit times, low variability among individuals, hydrophilic and hydrophobic drug loading, higher local drug concentrations, reduced side effects, low risk of dose dumping, possibility of different routes (e.g. oral, nasal, parenteral) of administration and also enhancing patient compliance (Mokhtare, 2015).

In the present study, STZ and NA were used to induce Type 2 DM in rats. After uptake by pancreatic β-cells, STZ causes increase in the production of free radical, reduced insulin synthesis in β-cells and also the death of β-cells. NA is used to prevent the STZ induced cytotoxicity. Thus, experimental Type 2 DM was induced in rats using this combination in the previous studies (Masiello *et al.*, 1998; Andersson and Sandler, 2001; Masiello, 2006; Tahara *et al.*, 2008; Szkudelska, 2012; Bisht and Bhattacharya, 2013; Ghasemi *et al*., 2014). STZ (50 mg/kg b.w.), and STZ (50 mg/kg b.w.) and NA (at different doses ranged of 0-200 mg/kg b.w.) combination were used to induce diabetes in rats in another study. The following results were reported: a). a severe reduction in serum insulin level of only STZ (50 mg/kg b.w.) induced-diabetic rats. b). when STZ-NA (50 mg/kg-100 mg/kg b.w.) combination was used to induce diabetes in rats, the severe reduction of fasting serum insulin level of rats was significantly limited and the rats' body weight loss was depressed by using NA. c). consequently, Type 2 DM was induced in rats by using STZ (50 mg/kg)-NA (100 mg/kg b.w.) combination (Tahara *et al.*, 2008).

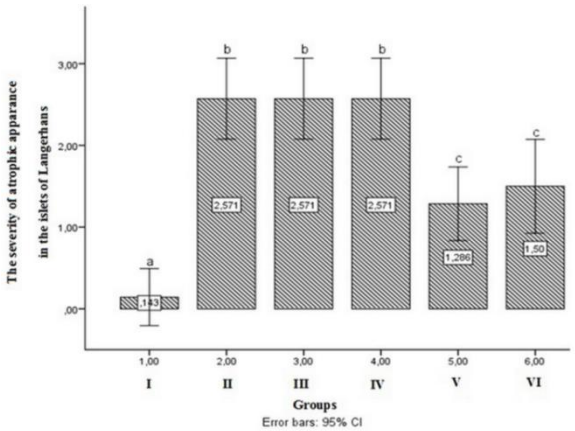

Fig. 3: The severity of histopathological changes in the experimental groups.

Table 1: The number of immunopositive β cells and the diameter of islet of langerhans in experimental groups

Groups	The diameter of islet of langerhans (μm)	The number of immunopositive β cells
I	45.15±3.41[a]	32.17±1.86[a]
II	11.04±0.60[b]	4.48±0.62[b]
III	12.61±1.84[b]	6.15±0.87[b]
IV	12.86±1.23[b]	4.53±0.55[b]
V	23.06±1.78[c]	13.01±1.78[c]
VI	25.57±0.95[c]	15.91±1.18[c]

[a,b,c] There was a significant difference among the groups signed with different letter in same column (P<0.05).

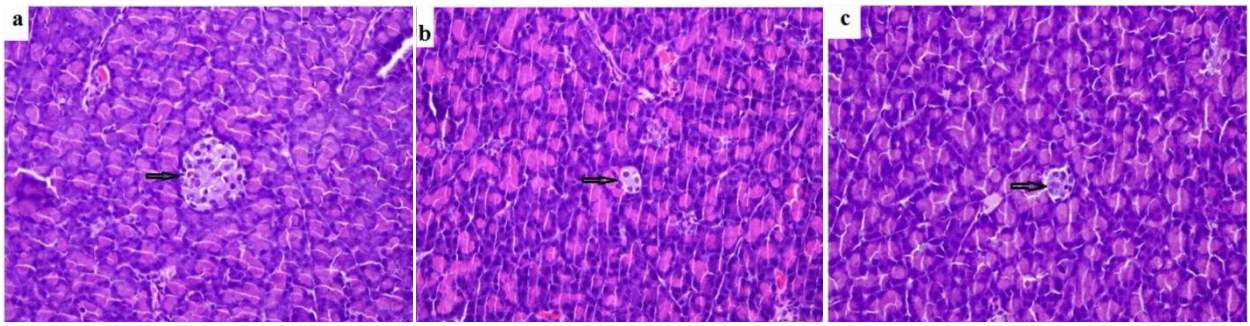

Fig. 1: a. Pancreatic section stained with Hematoxylin and Eosin of Group I; no atrophy in islets of langerhans (normal (-); arrow) (HE. 20µm). **b.** Pancreatic section stained with Hematoxylin and Eosin of Group II; the severe atrophy in islets of langerhans (+++; arrow) (HE. 20µm). **c.** Pancreatic section stained with Hematoxylin and Eosin of Group III; the severe atrophy in islets of langerhans (+++; arrow) (HE. 20µm).

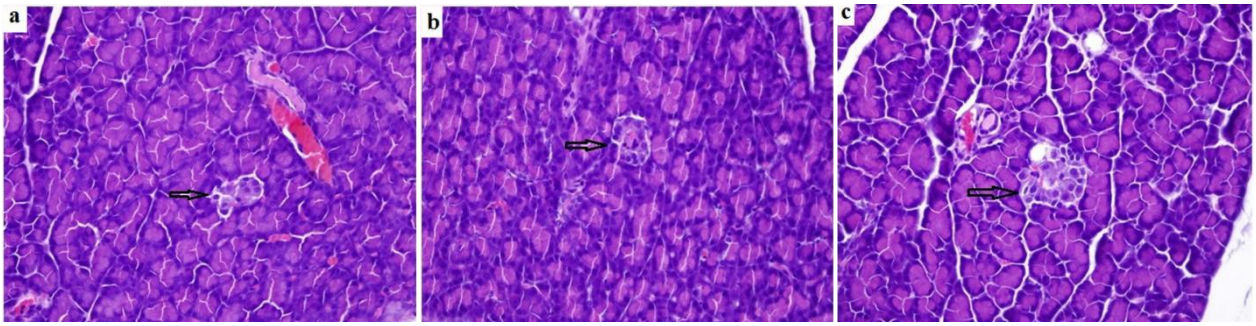

Fig. 2: a. Pancreatic section stained with Hematoxylin and Eosin of Group IV; the severe atrophy in islets of langerhans (+++; arrow) (HE. 20µm). **b.** Pancreatic section stained with Hematoxylin and Eosin of Group V; the mild atrophy in islets of langerhans (++; arrow) (HE. 20µm). **c.** Pancreatic section stained with Hematoxylin and Eosin of Group VI; the mild atrophy (++) in islets of langerhans (arrow) (HE. 20µm).

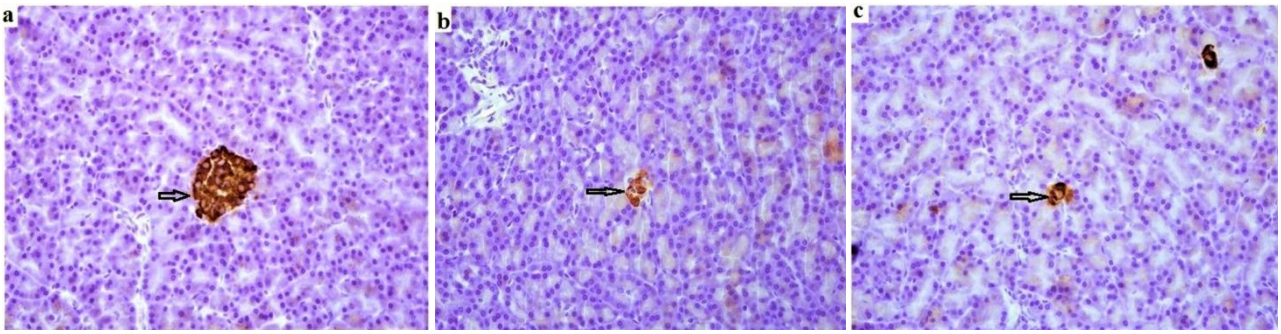

Fig. 4: a. The number of immunopositive β cells and the diameter of islet of langerhans of Group I. Normal diameter of islet of langerhans was observed (arrows) (IHC 20µm). **b.** The number of immunopositive β cells and the diameter of islet of langerhans of Group II. Shrunken and distorted islet of langerhans was observed (arrow) (IHC 20µm). **c.** The number of immunopositive β cells and the diameter of islet of langerhans of Group III. Shrunken and distorted islet of langerhans was observed (arrow) (IHC 20µm).

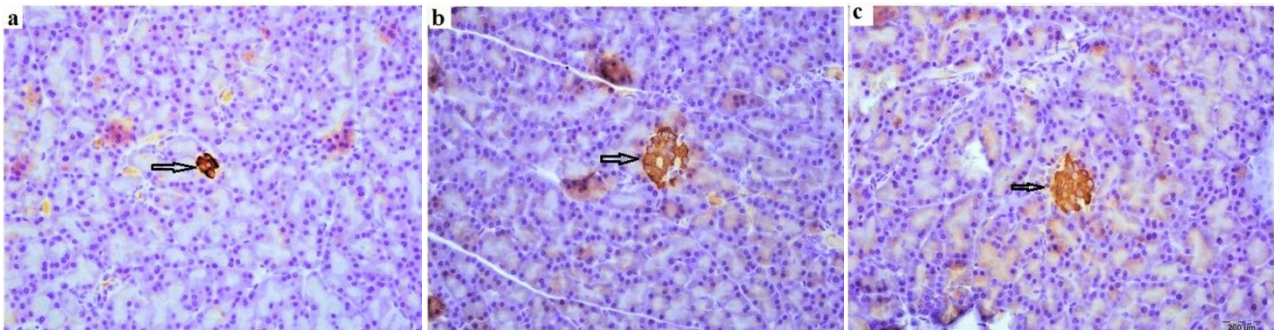

Fig. 5: a. The number of immunopositive β cells and the diameter of islet of langerhans of Group IV. Shrunken and distorted islet of langerhans was observed (arrow) (IHC 20µm). **b.** The number of immunopositive β cells and the diameter of islet of langerhans of Group V. The improvement of islet of langerhans was observed (arrow) (IHC 20µm). **c.** The number of immunopositive β cells and the diameter of islet of langerhans of Group VI. The improvement of islet of langerhans was observed (arrow) (IHC 20µm).

Nakamura *et al.* (2006) used STZ (100 mg/kg, i.p.) and STZ (100 mg/kg, i.p.) -NA (120 ve 240 mg/kg, i.p.) (STZ100-NA120/240) combination to induce Type 2 DM in mice. They reported that the pancreatic insulin content in diabetic mice administered with STZ100/NA120 and STZ100/NA240 was retained by 28 and 43% of healthy mice (control), respectively, and "histological damage of pancreatic beta cells was also less severe than that observed in STZ mice". And also, the histological damage of pancreatic β cells in diabetic mice administered with only STZ was more severe than that observed in diabetic mice administered with STZ100-NA120/240 combination. The combination of STZ and NA causes partial deficiency of pancreatic insulin.

Patanè *et al.* (2000) investigated the effects of metformin on pancreatic β cells in isolated pancreatic islets from rats. They found that metformin restores the normal secretary pattern in the islets of langerhans and it might have useful effects on the beta-cells secretory function.

And also, in another study, the effects of metformin on the histopathological changes of pancreas of rats with Type 2 DM-induced by using STZ (35 mg/kg, i.p.) were investigated. They reported that metformin (400 mg/kg per day) administered orally for 4 weeks has positive effects on insulin secretion and the regeneration of the langerhans islet beta cells (Ismail *et al*., 2015).

In addition, the effect of metformin was studied on developing pancreas and also beta cells by Gregg *et al.* (2014). When metformin was used during gestation, it regulates the initial steps of the development of β cell development and increases the number of pancreatic progenitors (Gregg *et al*., 2014).

In the current study, the degenerative and necrotic changes in endocrine part of pancreas issues of diabetic rats in Group II and diabetic rats treated with pure drug and blank beads in Group III and IV, respectively were observed (Fig. 1b - Fig. 2a, respectively). However, there was a reduction in the degree of necrotic and degenerative changes of islets of langerhans of diabetic rats treated with metformin HCl-loaded AL-CS and AL beads (Group V (Fig. 2b) and VI (Fig. 2c), respectively). Difference in the severity of atrophic appearance in the islets of langerhans was statistically significant between Group I and all other groups ($P<0.05$) **(**Fig. 3). There were no statistically significant differences in the severity of atrophic appearance of islets between Group II and III ($P>0.05$), and also between Group V and VI ($P>0.05$) (Fig. 3). However, the severity of atrophic appearance of islets had significant differences between the Groups (II and III) treated with pure drug/blank beads and the Groups (V and VI) treated with metformin HCl-loaded both beads formulations ($P<0.05$) (Fig. 3). It was determined that drug-loaded both bead formulations were more effective to reduce the histopathological changes of pancreas than pure drug ($P<0.05$).

In addition, the shrunken and distorted islet of langerhans with loss immunopositive β cells in Groups II, III and IV (Fig. 4b - Fig. 5a, respectively) was determined in immunohistochemical staining. There was a significant difference in the number of immunopositive β cells and the diameter of islet of langerhans between Group I and all other Groups ($P<0.05$) (Table 1). There were no statistically significant differences between the number of immunopositive β cells and the diameter of islet of langerhans of Groups II and III ($P>0.05$), and also those of Groups V and VI ($P>0.05$) (Table 1). On the other hand, difference in the number of immunopositive β cells and the diameter of islet of langerhans was statistically significant between the diabetic rats administered with pure drug/blank beads (Groups II and III) and the rats treated with metformin HCl-loaded AL-CS and AL beads (Groups V and VI) ($P<0.05$) (Table 1). Eventually, metformin HCl-loaded both bead formulations showed significant positive effects on the number of immunopositive β cells and the diameter of islet of langerhans of diabetic rats in Groups V and VI compared to the effect of pure drug ($P<0.05$).

Conclusions: It was determined that there was reduction in the number of pancreatic islet cells and the histopathological changes in pancreas of rats with diabetes induced by STZ-NA. Metformin HCl-loaded AL-CS and AL beads might be beneficial to reduce the histopathological changes of pancreas.

Authors contribution: YSS and MC designed the study and analyzed the data and also reviewed the results and content of the manuscript. BM executed the experiment and analyzed the data. All authors interpreted the data and approved the final version.

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