Relationship Between Structural Alterations of Ileal Smooth Muscle and Altered Responses to Acetylcholine in Streptozotocin-Induced Diabetic Rats[11]

STREPTOZOTOSİNLE OLUŞTURULMUŞ DİYABETİK SIÇANLARDA 'ILEUM DÜZ KASI YAPISINDA VE ASETİLKOLİNE CEVAPTA GÖZLENEN DEĞİŞMELER ARASINDAKİ İLİŞKİ

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Summary

Purpose: Although it is known that the gastrointestinal system is affected by diabetes mellitus, conflicting results have been obtained related with its function in diabetic animals. Functional studies in smooth muscles indicated that there was an increased number of muscarinic receptors associated with the increased contractile responses to muscarinic agonists. It is, therefore of interest to investigate the relationships between the increased contractile responses to muscarinic agonists and the structural alterations in the contractile performance of isolated ileal preparations of streptozotocin (STZ)-induced diabetic rats.

Materials and Methods: Wistar rats (200-250 g) were injected with single dose (50 mg/kg, i.p.) STZ (diabetic group, n=10) while the other animals were injected with the same amount of saline solution (control group, n=10). Experiments were performed after 4 weeks following a week of STZ injection. After the termination of 4 weeks, the ileal tissues were removed rapidly and the experiments were performed in both standard and Ca^{2+}-free Tyrode solutions. The maximum response to Acetylcholine (ACh) was obtained with 0.1 p.M ACh in control animals.

Results: The average peak tension of ACh-induced contractions of diabetic group recorded in both standard and Ca-free Tyrode solutions increased significantly with respect to the control values. In the histology of diabetic ileal tissues by both electron and light microscopies, we observed enlarged muscle cell size, an increase and dilatation in blood vessels, proliferative growth and vacuolated epithelial lining cells, and a tendency towards increased muscle thickness. Besides above finding, we noticed an increase in the number and size of the mitochondria, and in some areas rough surfaced endoplasmic reticulum.

Conclusion: These structural changes in the diabetic samples can partially explain the increased contractile responses of ileal preparations to the muscarinic receptor agonists.

Key Words: Smooth muscle contractility, Acetylcholine pre-contraction, Calcium, Structure, Ileum

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Özet

Amaç: Gastrointestinal sistemin diyabetten önemli derecede etkilendiği bilinmesine karşın, kimyasal yolla yapılan diyabetik hayvan modellerine ilişkin çelişkili sonuçlarla karşılaşılmaktadır. Diyabetik hayvanların düz kas fonksiyonunu açıklamaya yönelik çalışmalarda, muskarinik reseptör sayısında artışın olduğu, ve bunun sonucunda muskarinik agonist uygulamasının bu kaslarda kasılma kuvvetini arttırıcı yönde etki ettiği gösterilmiştir. Böylece bu çalışmada, muskarinik agoniste yanıt olarak artan kasılma kuvvetinin altında yatan nedenleri açıklayabilmek amacıyla, streptozotosin (STZ) ile oluşturulan diyabetik sıçan ileal preparatları yapısal değişmeler açısından araştırılmıştır.

Materyal ve Metod: Wistar sıçanlara (200-250 g) tek doz (50 mg/kg, i.p.) STZ enjekte (diyabetik grup, n=IO) edilirken diğer grup hayvanlara (kontrol grup, n=10) sadece salin enjeksiyonu yapılmıştır. Kasılma yanıtları ve yapı incelemeleri STZ enjeksiyonundan 5 hafta sonra yapılmış ve deney grubu hayvanların kan şekeri seviyeleri STZ uygulamasından bir hafta sonra ölçülmüştür. Beşinci haftanın sonunda hayvanların ileal dokuları hızlı bir şekilde çıkarılıp, kayıtlar hem standart hem de kalsiyumsuz Tyrod solüsyonu içinde yapılmıştır. Kontrol hayvanları için maksimum ACh yanıtı 0.1 pM'da elde edilmiş ve tüm ölçümler bu konsantrasyonda yapılmıştır.

Bulgular: Kontrol değerlen ile karşılaştırıldığında, ACh ile oluşturulan ortalama kasılma tepe değerlerinin (hem standard ve hem de kalsiyumsuz Tyrod çözeltilerinde) diyabetik grupta önemli derecede arttığı gözlenmiştir. Diyabetik İleum dokularında yapılan ışık ve elektron mikroskobu çalışmalarında, hücrede büyüme, artan ve dilate olmuş kan damarları, prolifère büyüme, vakuole olmuş epitel hücreler ve kas kalınlığında bir artma eğilimi olduğu gözlenmiştir. Yukarıdaki bulgulara ek olarak, hem sayıca hem de boyutça mitokondri artışı ve düzgün yüzeyli endoplazmik retikuluına rastlanmıştır.

Sonuç: Diyabetik dokularda rastlanan bu yapısal değişiklikler, muskarinik reseptör agonistlerine yanıtta gözlenen artışı kısmen açıklayabilmektedir.

Anahtar Kelimeler: Düz kas kasılması, Asetilkolin prc-kasılması, Kalsiyum, İnceyapı, Elektron mikroskopisi, İleum

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It is well known that as many other systems, the gastrointestinal system is also affected by diabetes mellitus. The diabetic patients seem to be prone to disorders of many systems such as cardiovascular (1), respiratory (2), urinary (3), and gastrointestinal (4,5). Vomiting, malabsorption, dysphagia, nausea, fecal incontinence and diarrhea are well known effects of diabetes in gastrointestinal system (4). Clarke and co-workers have shown that there was reduced peristalsis and dilation of the esophagus, gastric retention, disordered small intestinal movement, colonic atony and megacolon (6).

Clinical studies of intestinal function in diabetes mellitus have yielded conflicting results. Some results have shown rapid small bowel transit and diminished intestinal tone (7). Others have shown delayed transit through the ileum and increased intestinal motor activity (8). Microscopic studies of the myenteric plexus in the esophagus, and large and small intestine have either revealed no changes or abnormalities in the axons of extrinsic and intrinsic fibers (9).

Animal models of STZ-induced diabetes demonstrated megacolon (10), together with a deficit in the adrenergic and cholinergic innervation of the colon (11) and delayed small intestinal transit and a reduction in intestinal peristaltic activity (12). It has been shown that the small intestine undergoes anatomic and physiologic adaptive changes to be response to the diabetic state in humans and animal models. For instance, in animal models, the length and circumference of intestine were significantly increased (13). Korthuis and co-workers (14) have also demonstrated that, intestinal blood flow was increased along the length of the small bowel in diabetic rats relative to the control animals.

Although autonomic neuropathy is common in long-term diabetic patients and in experimental diabetes, the early effects of diabetes on muscarinic receptor processes have not been clarified adequately. Functional studies indicate an diabetes-induced alteration in the responsiveness of bladder smooth muscle to cholinergic agonists; however the results have been variable, with reports of both increased (15,16) and decreased (17) contractile responses. Latifpour et al., (16) reported an increased number of muscarinic receptors associated with the increased contractile responses to muscarinic agonists in the bladder dome from STZ-induced diabetic rats. Similar results were published by Carrier and Aronstam (18) in which they observed greater contractile force in response to muscarinic agonists of diabetic ileal preparations than in controls while there was a 32% reduction in the density of muscarinic receptors of muscles from diabetic rats and there was no change in agonist binding affinities in muscles from diabetic rats.

In vitro experiments have also revealed diabetic changes in the gastrointestinal tract. Decreased P-adrenergic responses have been observed in the different segments of small intestine such as duodenum (19-22) and ileum (23) from rats with ALL- and/or STZ- induced diabetes.

Although the results about gastrointestinal tract in diabetes mellitus are conflicting with each other, there are yet not enough data to demonstrate the origin of alterations observed in diabetes mellitus status. Histochemical studies of the ileal myenteric plexus from STZ-induced diabetic rats revealed degenerative changes in adrenergic and serotonergic nerves (24). In contrast, cholinergic nerves did not display signs of degeneration. It is therefore of interest to investigate the relationship between the increased responses to ACh of isolated rat ileum and the structural alterations under STZ-induced diabetes in rats.

Materials and Methods

Adult Wistar rats in both sex, weighing between 200-250 g were used for both control (n=10) and diabetic (n=10) groups. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ, Sigma Chemical, 50 mg/kg) delivered in a citrate-buffered vehicle, pH 4.5 (25). Control animals received a similar injection of the vehicle alone. All animals were fed with normal rat chow and water ad lib and were sacrificed 4 weeks after STZ injection. Animals were tested for diabetes 7 days following the injection by checking blood (from tail) glucose level using glucometer (Roche). Body weight and blood glucose was monitored as

indicators of diabetes. Blood glucose levels of the animals were measured second times at the termination of the experiment.

At the termination of the experiment, all animals were weighted and anaesthetised lightly with pentobarbital (30 mg/kg body weight) intraperitoneally. The abdomen of the rat was opened and the ileum was removed immediately. Then it was cleaned free of its contents and flushed with ice-cold Tyrode solution, and 1-cm length of tissue suspended in 40 ml tissue baths containing Tyrode solution of the following composition (mM): NaCI 137, KCI 2.7, $MgS0_4$ 1.4, $CaCI_2$ 1.8, $NaHC0_3$ 11.9, NaH_2P0_4 12, glucose 5.5. Tyrode solution was bubbled continuously with 95% 0_2 and 5% $C0_2$ gas mixture, and maintained at 37° C and pH of the medium was 7.4.

To record tension, samples were mounted vertically between a fixed metal holder, and a force transducer (Ugo Basile isometric transducer No.7003) and the resting tension of each sample was adjusted to 0.3 g. The tissue preparations were allowed to equilibrate for 30-40 minutes before the experiments. The isometric tensions were recorded on a paper chart recorder (Ugo Basile No.7050). After equilibration, the ileum pieces were precontracted with ACh added into bath solution and the maximal response was obtained with a concentration of 0.1 uM ACh. In the second part of our experiment, Ca^{2+}-free solution was prepared by equimolar substitution of $CaCI_2$ with NaCI (26).

In order to investigate the histology of ileal tissue by using light microscopy, the ileal pieces were fixed into 10 % formalin buffered by 0.067 M phosphate pH 7.2 for 24 hours by changing the formalin two times and were paraffin embedded for histological sectioning and were stained with haematoxylin-eosin and Masson's trichrorne staining. Micrographs were taken with a Carl Zeiss photomicroscope (27).

Ultrastructural investigations were performed by using a Jeol 100 transmission electron microscope. Tissue specimens fixed in 2.5% glutaraldehyde in a phosphate buffer (pH 7.2) for 24 hours. Pieces were then post fixed in 1% osmium tetroxide. The materials were dehydrated in graded alcohol, embedded in Araldite CY212, sectioned with a L K B Ultratome III, and stained with uranyl acetate and lead citrate (27).

A11 chemicals used were of analytical reagent grade. Values presented are meansiSD. Statistical significance was assessed with Student's t-test.

Results

The mean weights of diabetic group animals were significantly lower than that of the controls. The mean blood glucose level was significantly higher (around four times of the control) than the control blood glucose level at the termination of the experiment ($p<0.05$). No other significant changes were observed in these diabetic animals.

The effect of diabetes mellitus on the ACh precontracted ileal response in standard and Ca^{2+}-free Tyrode bath solution is summarized in Figure 1. The responses are presented as percentage. The responses to different ACh concentrations between 0.001-0.1 uM were examined and the maximal response to 0.1 j.iM ACh of control samples is accepted as a 100% response and the responses of experimental samples to the same ACh concentration were calculated in both standard and Ca^{2+}-free Tyrode solution. The responses of diabetic ileal preparations to 0.1 ***xM** ACh were 217±34% and 146±35% in standard and Ca^{2+}-free Tyrode solu-

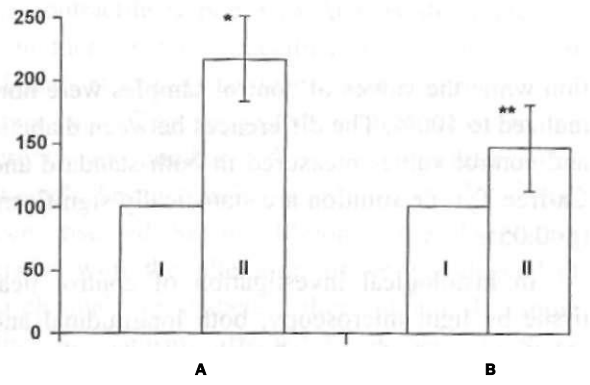

Figure 1. The maximal responses to acethlycholine (ACh) of isolated ileal preparations of the control (l) and the diabetic **(II)** rats recorded in both normal (A) and Ca^{2+}-frec (B) Tyrode bath solutions. The maximal response (100%) to ACh of the control preparations was obtained with 0.1 pM ACh concentration in normal Tyrode bath solution (*$p<0.001$, **$p<0.01$).

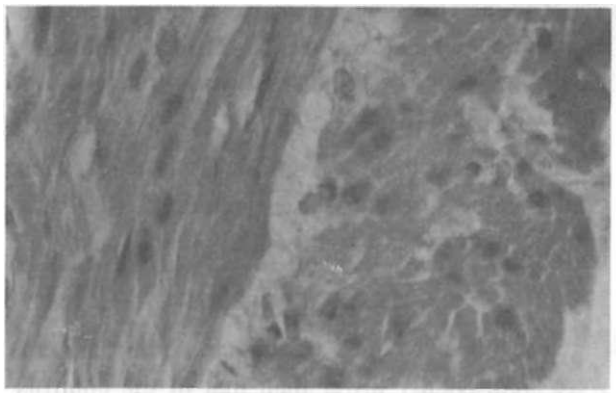

Figure 2. Light microscopy of control group. Smooth muscle layer of ileum is showing circularly and longitudinally oriented cells. H.E. X250.

Figure 3. Light microscopy of the diabetic group. Photomicrograph of longitudinally sectioned smooth muscle of the ileum showing an increase in the cell size and elongated nuclei. H.E. X300.

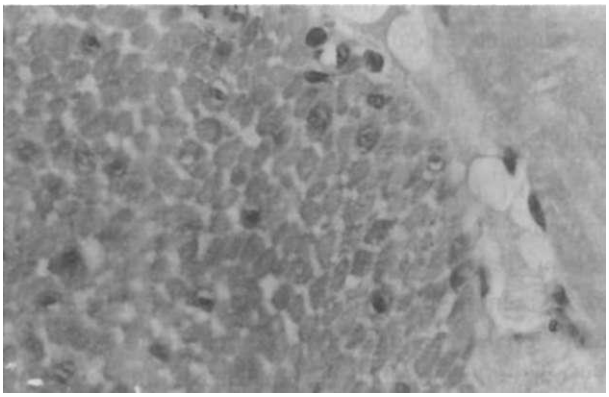

Figure 4. Transverse profiles of smooth muscle layer of the diabetic group The muscle cells appear larger and separated by enlarged (dilated) intracellular space. X300.

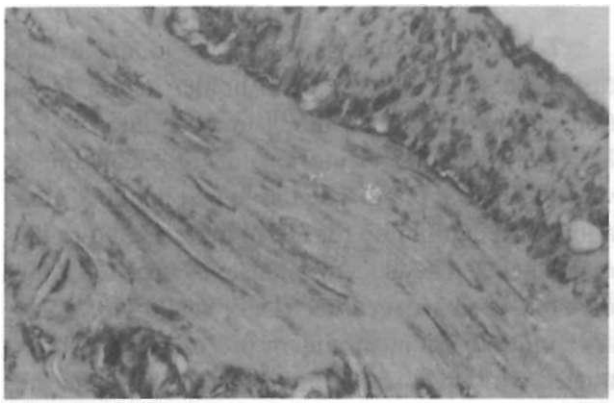

Figure 5. An increased and dilated capillaries in the muscle layer are seen in diabetic group. Masson's Trichrome stain. X150.

tion while the values of control samples were normalized to 100%. The differences between diabetic and control values measured in both standard and Ca-free Tyrode solution are statistically significant (¿0.05),

In histological investigation of control ileal tissue by light microscopy, both longitudinal and circular muscle layers of tunica muscularies are seen in Fig. 2. In this figure, longitudinal layer cells are elongated with central elongated nuclei and homogeneous cytoplasm and ill-defined borders whereas only a few of the transverse profiles show nuclei and cell lie closely apposed with little intercellular material (Figure 2). In experimental group, a tendency towards increased muscle thickness are noted. Smooth muscle cells both of sectioned, as longitudinally and transversely, show enlarged cell size and dilated intercellular space (Figure 3 and 4). An increase and dilatation of blood vessels and capillaries in the muscular layer of this group are noticed (Figure 5). In diabetic samples, the mucosa of ileal tissue is also characterized by proliferative growth and vacuolated epithelial lining cells. In ultrastructural investigation of the groups, the size and the number of mitochondria of the muscle cells of the diabetic group

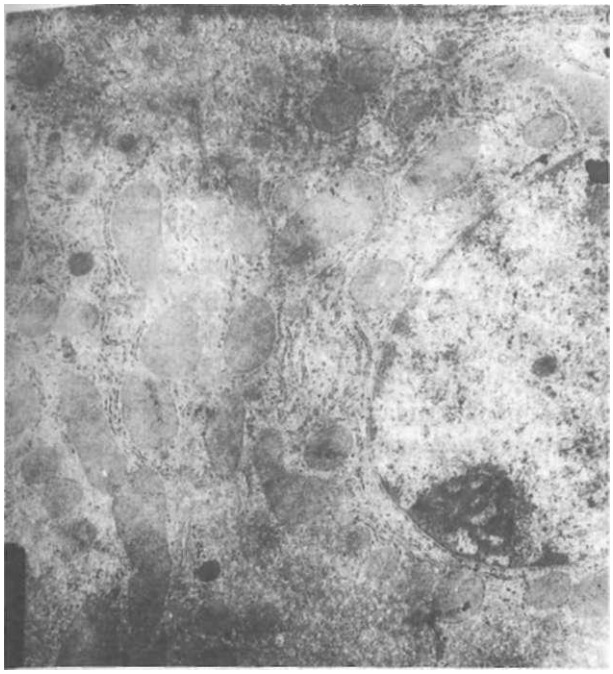

Figure 6. Election micrograph of a muscle cell of the diabetic group. The mitochondria are enlarged in size and number. A close association of mitochondria with rough-surfaced endoplasmic reticulum and many free ribosomes are seen. Bar= 1 pm.X19.000.

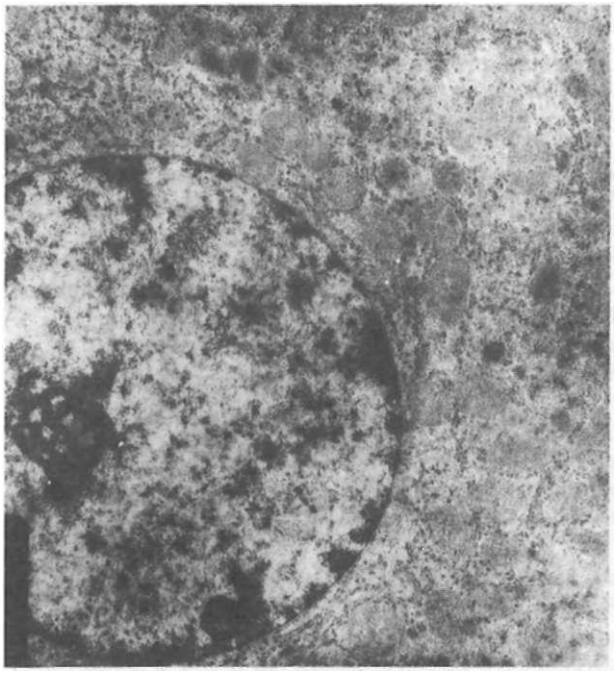

Figure 7. Electron micrograph of sarcoplasm at one of the nuclear poles of smooth muscle cells from the control group. Within the sarcoplasm, there are smooth and rough-surfaced elements of the endoplasmic reticulum, ribosomes, and mitochondria. Bar= 1 pm. XI 5.000.

are increased (Figure 6) with respect to the control group (Figure 7). A prominent close association are seen between rough endoplasmic reticulum and mitochondrion (Figure 6).

Discussion

In our experimental diabetes models of rats by injection of streptozotocin (STZ), the maximal ileal response to acethylcholine was increased significantly with respect to the control response in both standard and Ca^{2+}-free Tyrode solution. Carrier and Aronstam (18) studied ileal smooth muscle samples of rats rendered diabetic by STZ injection and they showed that these muscle samples developed greater contractile force in response to muscarinic agonists (ACh etc.) than muscles from aged-matched control rats. These investigators concluded that although there was a 32% reduction in the density of muscarinic receptors in the diabetic group, the origin of the increased responsiveness of the preparations in this model is not clear but involves changes in the muscarinic signal transduction pathway beyond the receptor level.

In the present study we tried to investigate the other possible sources of the increased contractile responsiveness to ACh of ileal preparations. For this aim first we tried to find a relationship of it with Ca-homeostasis and we used Ca^{2+}-free bathing medium and repeated the all experiments. Our data measured in Ca^{2+}-free bath solution show that the contractile response to ACh is still increased even there is no extracellular Ca^{2+}. The data of Aighara et al (28) confirmed our observations in some part. They suggested to an increase in the activity or number of voltage-dependent Ca-channels, because an increased influx of $^{45}Ca^{2+}$ has been observed. So, in addition to the observation related with the alteration of voltage-dependent Ca-channels in diabetes, there should be some other mechanisms affected by diabetes in these types of tissues.

The histological investigation of diabetic ileal tissue shows that diabetes caused an increase in muscle thickness, an increase and dilatation of capillaries, and increases in the size and number of

mitochondria. These structural alterations in diabetic ileal preparations indicate that diabetes mellitus can induce an increase in the blood and energy need of the ileal tissue and this increased necessity is compensated with alterations in the structure of these samples. This structural alteration observed in the ileal samples of the diabetic rats can partially explain the increased contractile responsiveness of them to the muscarinic receptor agonists.

Previously published studies have shown some degenerative changes and damage as well as some degenerative enteric ganglion cells in ileal tissue under experimental diabetes in animal models. Despite of many researches in the field of the effects of diabetes mellitus on intestinal morphology and function, little is known about the effects of diabetes on the anatomy and physiology of intestinal smooth muscle. In the study of Lincoln et al (24), it was demonstrated that although histology of the cholinergic nerves did not show any signs of reduction, choline acetyltransferase and acetylcholinesterase activities per centimeter of intestine are increased. Also it was shown that there was no change in acetylcholinesterase activity in muscle membrane isolated from STZ-diabetic rats (18). Under the studies performed up to now, together with the present data, we can conclude that there should be some changes in the sensitivity of diabetic smooth muscle to autonomic agonists besides the structural changes in these muscle cells. These types of changes might be specific to region of tissue and related to the duration of the diabetic state. In the study of Kolta et al (15), after 6 weeks of streptozotocin-induced diabetes, muscle from the base of the rodent urinary bladder showed a 39% increased contractile response to ACh, where as muscle from the bladder body remained unaffected. On the other hand, in the same experiments, after 47 weeks of diabetes, the bladder body showed the same enhanced sensitivity to ACh. These results showed that the duration of diabetes is important in the appearance of insets. In a recent study by Jorgensen et al. (29), tension-strain relations and morphometry of rat small intestine in 4 week experimental (STZ) diabetes were investigated. They have shown that the tension-strain relations in jejunum and distal ileum were nonlinear and the curves for the diabetic group was shifted to the left compared to the curve for controls, indicating increased wall stiffness.

One can give several factors altering the effects of diabetes on the function and structure of tissues and one can observe some controversy in the results. The duration of diabetes, the experimental periods to perform experiments after induction of diabetes, the age and the sex of animals, the region of investigation on the tissue, and also the techniques to measure the changes are between the reasons for this controversy in the gastrointestinal cholinergic responsiveness. Our experimental period was 4 weeks starting from the injection of STZ. This period should be not enough to observe a fully alteration in both function and morphology of this tissue. Some of the studies were carried out 4-7 months after induction of diabetes with STZ but our animals started to die after ***5-6*** weeks of induction. Recently, Verhofstad et al. (30) were studied the mechanisms that cause diabetes to impair the development of anastomotic strenght in the intestine in rats. They have shown experimental diabetes lead to alterations in cellular components involved in the early phase of repair of intestinal anastomoses but not to a reduced accumulation of wound collagen. Although, in the study of Horowitz et al (31), it was demonstrated that the blood glucose concentration modulates the perception of some sensations arising from gastrointestinal tract, the underlying mechanisms are yet not clear and needs further studies.

All these observations clearly indicate that the side effects of diabetes are dependent on the duration and the type of experimental procedure performed in the animals as well as their species and sex. In gastrointestinal smooth muscle, the duration of diabetes seems to be effective in the contractile machinery of this system.

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