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SMOOTH MYOCYTES AND COLLAGENOUS FIBERS OF THE URINARY BLADDER OF RATS IN DIABETES MELLITUS

Tokaruk Nadiya

Department of Human Anatomy, Operative Surgery and Topographic Anatomy
Ivano-Frankivsk National Medical University, Ivano-Frankivsk, Ukraine

Key words: diabetes mellitus; smooth myocytes; collagenous fibers.

Introduction. Diabetes mellitus (DM) causes diabetic cystopathy, which is associated with detrusor dysfunction and the content of collagenous fibers. The results of the performed studies are ambiguous and often contradictory, requiring objective data which could be obtained on the basis of the simultaneous determination of relative areas of smooth myocytes and collagenous fibers and their ultrastructural study.
Objective: To determine the peculiarities of the structural and metric organization of smooth myocytes and collagenous fibers of the urinary bladder (UB) of rats during different stages of DM.
Materials and methods. DM was modeled by streptozotocin in Wistar rats. Relative areas of the studied structures were defined on digital images of histological sections of UB stained by Mason using the original automatic way. Smooth myocytes were studied ultrastructurally.
Results. During the 14^{th}-28^{th} day of DM development the percent of collagenous fibers area decreases and the percentage of smooth myocytes area of UB wall increases. The expanding of intercellular spaces and the development of vacuolar degeneration of myocytes are observed. During the 42^{nd}-56^{th} days the percentage of collagenous fibers area increases and the percentage of the area of smooth myocytes decreases. Ultrastructurally subsiding of vacuolar dystrophy, short-term baloon dystrophy, the appearance of dark myocytes, moderate karyorrhexis were observed. During the 70^{th} day of the experiment the percentage of collagenous fibers and smooth myocytes areas does not change significantly, most dark myocytes are involutive, there are local fibrosis and myocyte sequestration areas.
Conclusions. Ultrastructural changes are characterized by a pronounced polymorphism and have a chronological relationship. Author's worked out original method of determination of the relative area of structural UB wall components determines entirely new opportunities to perform morphometric analysis of UB.

1. Introduction.

Diabetes mellitus (DM) causes diabetic dysfunction of the urinary bladder (UB), which is associated with dysfunction of the bladder detrusor, which leads to the change in its urinary capacity, and with the content of collagenous fibers that provide suppleness of UB wall to stretch. We have found that the results of studies on these issues are different.

Thus, some authors have established the increase of contractile ability of UB in DM [6, 24], and others – the decrease of it [17, 22, 33], and still others [12] indicate that DM can both decrease and increase the detrusor contractility. Several authors argue that in the early stages of DM development (3^{rd} and 9^{th} weeks) contractility of the UB increases, and during the later periods (12^{th} and 20^{th} weeks) – it decreases [7, 8, 14]. They suggest that in rats and mice the transition to decompensated stage of UB dysfunction occurs during the 9^{th} – 12^{th} weeks of DM.

Implicitly, these changes of urinary ability of the UB are accompanied by the restructuring of smooth myocytes. Thus, the researchers observed detrusor hypertrophy during the 3^{rd} [19], 4^{th} [21], 5^{th} [24, 36], 8^{th} [18] and 3^{rd} – 9^{th} [39] weeks and in 6 months [15] form the beginning of DM development, and A.A. Rodrigues et al. [27] did not observe the detrusor hypertrophy in alloxan diabetes.

Collagenous fibers are the main component of extracellular matrix of UB and affect passive biomechanical properties of its wall [19]. C. C. Wang et al. [38] examining the biomechanical properties of the UB strips, have found that streptozotocin-induced diabetes increases its susceptibility to tissue stretching during the 4^{th} – 8^{th}-weeks of DM development, and G. Liu et F. Daneshgari [19] consider that the percentage decrease of the area of collagenous fibers points to the increased extension of UB in DM. The growth of UB plasticity in DM is pointed by other authors [10]. However, there are works which determined that collagen and the percentage of fibrosis area to the total area of the UB wall in DM do not change [18, 27].

Presence of ambiguous and often contradictory results of studies performed in different laboratories, at different times of DM, with various animal species, age and gender, with different approaches to diabetes modeling and morphometry performance, requires further research to give objective and irrefutable data which can be only obtained based on morphometric studies of structural components of UB wall.

To realize the above said they often use thickness of different layers of UB wall [11, 17]. However, this index can be applied only to determine the total UB wall thickness and urothelium thickness, because only they have clear boundaries. More objective is to determine the components of the wall area. However, the method of determination of the areas which "manually" or automatically are fortified by the boundary line [18, 37], is possible only for determination of the area of urothelium, which is the epithelial tissue, which contains almost no intercellular substance. Thus, the received values of the mentioned areas of collagenous fibers and smooth myocytes cause significant reservations because their area is calculated, in this case, together with the main substance of the extracellular matrix, the content of which varies considerably in swelling or dehydration of these structures.

The above mentioned drawbacks are absent in determination of the areas of structural components of the UB wall on digital images of histological sections stained by trichromatic method according to Mason [28], and the further use of colour segmentation. This staining makes it possible to visualize smooth myocytes and collagenous fibers in red and blue colours. Thus, the main substance of the extracellular matrix is not stained. It's necessary to note that the staining of histological sections according to Mason is used by many researchers [10, 15, 19, 24, 27], but the colour segmentation – only by some of them [19, 24]. However, applications for morphometry that allow to perform segmentation, have a significant drawback – the user sets the boundaries of colour values for each component of the image "manually", that brings considerable subjectivity on the obtained data.

Objective: to determine the peculiarities of the structural and metrical organization of the smooth myocytes and collagenous fibers in rats' UB at stages of DM development.

2. Materials and methods

2.1. Animals and Experimental Model

Experiment was performed according to the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The study was performed using 80 adult 1-year-old male rats of Wistar line: 10 of them were intact (normal); 50 (10 on each observation period) were modeled diabetes mellitus by intraperitoneal administration of streptozotocin (60 mg/kg of body weight) dissolved in 0.1 M citrate buffer; 20 were control rats (4 per each term), they were injected only citrate buffer intraperitoneally. Collection of the material was performed during the 14^{th}, 28^{th}, 42^{nd}, 56^{th} and 70^{th} day of DM. Diabetic rats were considered only those who had glucose level at least 12 mmol/l.

2.2. Methods of morphological research

Histological sections were stained using trichromatic method according to Mason [28]. Electronic-microscopic examination was performed according to the appropriate recommendation [29].

2.3. Morphometry

Determination of the relative areas of the studied components of the UB wall was performed in ImageJ v. 1.47 (NIH, USA, http://imagej.nih.gov/ij) [30] using the original method [34], which allows us to represent the area of smooth myocytes, collagenous fibers and urothelium in percentage of their total area.

2.4. Statistics

There were used non-parametric statistical methods (Wilcoxon-Mann-Whitney test), which were performed in R v. 3.0 [25].

3. Results of the study and their discussion.

The structure of the muscular layer of rats' UB microscopically and ultrastructurally do not significantly differ from that, which is described in the classic work of A.W. Ham et Cormack D.H. [13]. According to the results of our research work we can add that light smooth myocytes are most common in control rats (Fig. 1a). Organelles of these cells are near the nuclei poles. Mitochondria are also well-placed across the myocyte's section and near its plasmolemma. They are light, with crests, different in shape and small and medium in size. Cisterns of granular endoplasmic reticulum are short, and they are few. Golgi complex is represented mostly by short and narrow tubes and small, rarely medium sized vesicles. Near the mitochondria beta-granules of glycogen that look less electronically dense and larger in size compared to free ribosomes, are located in a small amount. Free ribosomes are many in number. There is also moderate amount of caveolae and pinocytic vesicles. There are some areas where one can see the smooth myocytes of different electronic density, and the organelles, especially mitochondria, can be distinguished in each of them (Fig. 1 b).

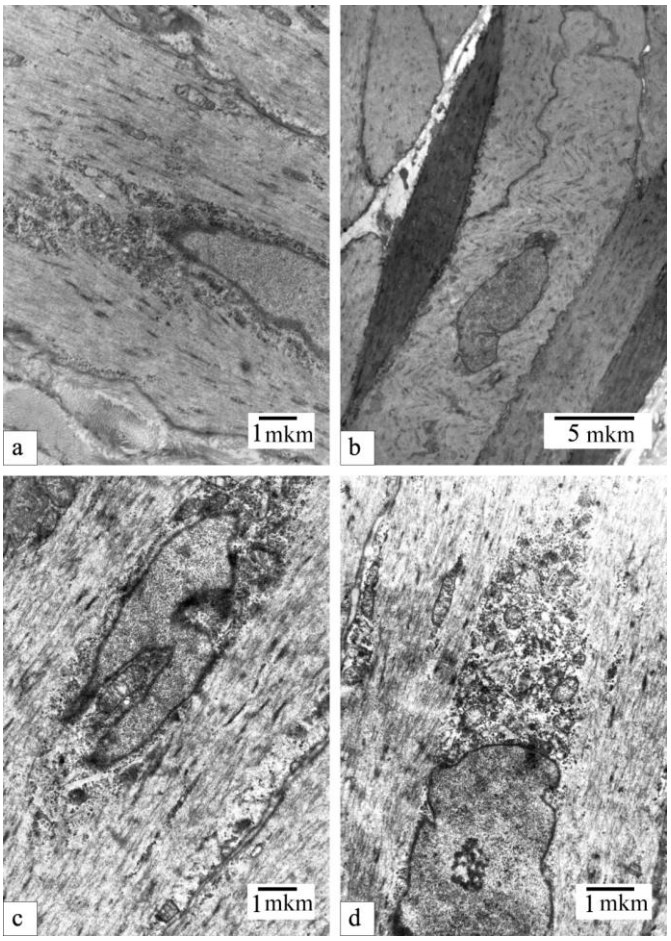

Fig. 1. Ultrastructural structure of smooth myocytes of the control rats (a, b) and myocytes, which are in the state of functional tension during the 28^{th} (c) and 42^{nd} (d) day of diabetes mellitus. Transmission: electron microscope images.

The question of light and dark smooth myocytes of the visceral organs are repeatedly discussed in the literature. Essentially there are two different opinions. Some authors [32] believe that these cells differ in their degree of differentiation: the light ones that are rich in organelles of biosynthesis,– are the myocytes that could be differentiated and dark ones that are rich in contractile apparatus,– are highly differentiated cells. Other researchers [40] indicate that both types of smooth myocytes have a high degree of differentiation and they are not significantly different by the structure of mitochondria, endoplasmic reticulum, nuclei ultrastructural organization. These two types differ only by spatial arrangement of myofilaments. In the dark myocytes they are located tightly and parallelly, and in light ones – more or less loosely sorted. Moreover, light myocytes were observed to have many caveolae of subplazmolemmal pinocytic vesicles that provide transportation, concentration and excretion of Ca^{2+} [40]. These authors argue that light and dark smooth myocytes are at different phases of functional activity. Thus, not all light cells are involved into the active reduction, because some of them are functional reserve. According to our research we are more inclined to the second opinion, but pinocytic vesicles are observed also in the dark myocytes.

We have investigated the collagenous fibers that make up the bulk of the fibrous extracellular matrix structures and they may cause UB ability to increase its volume, which is important in polyuria that develops in various forms of diabetes mellitus. Thus, special attention was focused on the changes in the area occupied by collagenous fibers.

Digital images of histological sections stained according to Mason at the stages of DM progress are represented in Figure 2, and the results obtained using the method developed by us are in Table 1.

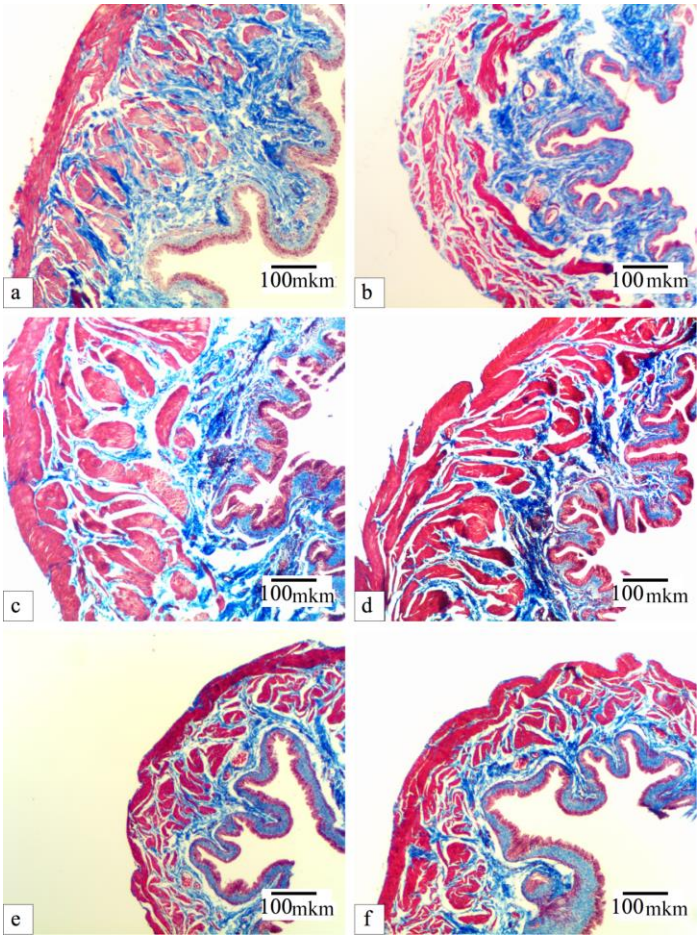

Fig. 2. Smooth myocytes (red colour) and collagenous fibers (blue colour) on the equatorial sections of UB wall in the control rats (a) during the 14th (b), the 28th (c), 42nd (d), 56th (e) and 70th (f) days of DM development. Staining: trichromatic method after Mason. Magnification: × 10.

Table 1

Results of morphometric study of the structural components of the urinary bladder wall using colour segmentation at the stages of streptozotocin-induced diabetes (Mean ± SD)

Terms of the experiment (day)	The percentage area (%) of the studied structure the UB wall to the total area of urothelium, collagenous fibers and smooth myocytes	
	collagenous fibers	smooth myocytes
control rats	38.57 ± 6.13	53.81 ± 6.66
14th	33.74 ± 3.37$_{\#\#}$	55.91 ± 5.56
28th	20.85 ± 3.02$_{\#\#\#}^{***}$	68.15 ± 4.15$_{\#\#\#}^{***}$
42nd	23.29 ± 4.77$_{\#\#\#}^{*}$	67.54 ± 5.19$_{\#\#\#}$
56th	28.87 ± 5.35$_{\#\#\#}^{**}$	62.89 ± 5.62$_{\#\#\#}^{**}$
70th	32.36 ± 5.43$_{\#\#\#}$	60.76 ± 5.43$_{\#\#\#}$

Notes: Mean ± SD – average value ± standard deviation; statistically significant difference with indicators of control animals - $_{\#\#}$ ($p<0.01$), $_{\#\#\#}$ ($p<0.001$) and previous period – * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$).

The determined by us areas of studied UB wall components of the control animals, are similar to those in the work of G. Liu et al. [19], and according to the results of other authors [24] – only according to the percentage of the area of smooth myocytes. In recent authors myocytes' areas percent is greater than our data, and the percentage of collagenous fibers area – less one, that most likely is connected to the subjective factor, as the authors performed segmentation "manually", and we – automatically. Thus, the dynamics of hypertrophy of smooth myocytes, which was also identified in the streptozotocin-induced diabetes, is similar to the data of G. Liu et al. [19], and D. A. Pitre et al. [24]. The first ones used 10-week old rats and the greatest hypertrophy of detrusor was found on the 3rd week of DM, in the others – rats' age was higher than a year and the highest level of hypertrophy was observed at the 5th week,and we have taken the 12-month-old rats and the highest detrusor hypertrophy was observed during the 28th day of the experiment. These differences are explained by the age of animals – the younger rats, the more rapid is the detrusor hypertrophy [19].

We have determined that compared to the control animals the percentage of smooth myocytes area begins to grow from the 14th day of DM and during the 28th day statistically significantly increased 1.28 times, which coincides with their biggest daily urine output, which exceeds such one in control animals 15.8 times ($p<0.001$) [35]. During the 42nd day the percentage of smooth myocytes area of diabetic rats is not statistically significantly reduced from the previous period, despite the fact that their daily urine output decreases 1.45 times ($p<0.01$). During the 56th and 70th days of DM development the percentage of the smooth myocytes area of the studied rats was reduced, compared with the 28th day only 1.08 ($p<0.01$) and 1.12 ($p<0.001$) times, while at the same time daily urine output of these rats decreased 2.1 times ($p<0.001$). As is evident from the above said, in DM there is definite dependence between rats' diuresis and UB detrusor hypertrophy. Our data coincide with the data of other researchers who believe that the smooth muscle hypertrophy of the UB in DM stimulates the increased urine output several times [8, 24, 36]. However, we have noticed some lag of hypertrophy regression of smooth myocytes on the reduction degree of daily urine output.

In our study (Table 1) the percentage of the area of collagenous fibers of the UB wall firstly decreases progressively and during the 28th day of the experiment it becomes smaller than the same in control animals 1.85 times. ($p<0.001$). Reduction of the percent of collagenous fibers area in the early stages of streptozotocin-induced diabetes other researchers have also observed using the method of segmentation, namely: G. Liu et F. Daneshgari [19] – during the 3rd week and D.A. Pitre et al. [24] – during the 5th. In the following two terms there is the reverse process and the percentage of collagenous fibers area during the 56th day, compared to 28th day, statistically significantly increases 1.38 times. After 10 weeks from the beginning of DM development the percentage of collagenous fibers area, as the percentage of smooth myocytes area, does not differ from the previous period ($p>0.05$), but the value of the first parameter is less than in the control group 1.19 times ($p<0.001$), and the value of the second one – 1.13 times greater ($p<0.001$). The received data during the 10th week of the experiment are similar to the results that G. Liu et F. Daneshgari [19] have obtained during the 9th week of DM development.

Considering that up to the end of the experiment smooth myocytes stay hypertrophied, which indirectly indicates the ability to preserve urinary ability of the UB [14, 19], and also the fact that the percentage of collagenous fibers area is lower than the control and there is no reason to speak about diffuse fibrosis of the UB wall, we can say that rats' diabetic cystopathy is compensated by the end of the experiment, which we have performed. Our conclusion is not contrary to the assertion of several authors [7, 8, 14] that in the streptozotocin-induced diabetes the UB dysfunction in rats and mice passes into the decompensated stage during the 9th-12th weeks of its course. Support of our conclusion objectivity is also indicated by the results of studies in which the reduction of urinary ability of the UB in diabetic rats was found

only in 6 months [5], and the increase of fibrosis area – in 6 [15] and 11 [27] months of DM induction.

We have determined that during all the terms of observations there are smooth myocytes in the UB, which do not significantly differ from those of control rats by ultrastructural structure. In addition, during the development of DM smooth myocytes occur, which are in a state of functional tension (Fig. 1c, d), which is confirmed as the authors indicate [2, 31] by their large nuclei, numerous protrusions of nuclear membrane, increase in the size and number of mitochondria, which are located near the nucleus, a large number of free ribosomes and glycogen inclusions. According to our data, such myocytes are light and have increased content of the distinct myofilaments placed densely in the cytoplasm and parallelly in one direction.

They could be observed most often during the 28^{th} and 42^{nd} day of the experiment. Hypertrophied UB smooth myocytes with a high content of glycogen granules and free ribosomes were also observed in benign prostatic hypertrophy [20, 23]. Dark myocytes with every term of diabetes development are identified more often, as indicated by other authors [26]. We have determined that these cells are of two types. In some – organelles, pinocytic vesicles and myofilaments are preserved (Fig. 3a). These myocytes are referred to the dark smooth functioning myocytes, which are also observed in control rats. Other dark myocytes have often vacuolar osmiophil cytoplasm, few mitochondria with crests destruction, poorness of other organelles and lack of pinocytic vesicles, a few myofilaments (Fig. 3b). These dark myocytes are referred to the involutive ones which are in a state of necrobiosis. The content of the first ones with the development of DM over time is decreased, and of the second ones – is increased. Similar by ultrastructural construction smooth myocytes were observed in UB also in benign prostatic hypertrophy [20, 23] and DM in rats [26].

During the 14^{th} day from the beginning of diabetes mellitus the most characteristic changes in the smooth muscular tissue of UB is the expanding and lumen of intercellular space, indicating swelling of the intercellular substance. During the 28^{th} day the most important is the development of vacuolar degeneration of smooth myocytes, manifestations of which are different. Thus, in some myocytes there is the expansion of perinuclear space, swelling and destruction of mitochondria, and along with the destroyed ones – there are many mitochondria of the conventional structure (Fig. 3c). Others – have vacuolization and expansion of bags and cisterns of granular endoplasmic reticulum and the tubules and vesicles of Golgi apparatus (Fig. 3d). During the 42^{nd} day of the induction of diabetes the manifestations of vacuolar dystrophy in muscular tissue of the UB do not subside, and in some myocytes there is karyorrhexis (Fig. 4a). The most characteristic of this period is the appearance of large blisters in smooth myocytes – baloons which compress organelles or nucleus (Fig. 4b). 56^{th} day differs from the previous period by subsiding of the effects of vacuolar and baloon dystrophies and more pronounced karyorrhexis. During the 70^{th} day of DM muscular membrane of the UB is characterized by the absence of phenomena of vacuolar and baloon dystrophies. Most dark smooth myocytes are of involutive type. There are areas that can be interpreted as focal fibrosis (Fig. 4c) and sequestration of smooth myocytes (Fig. 4d).

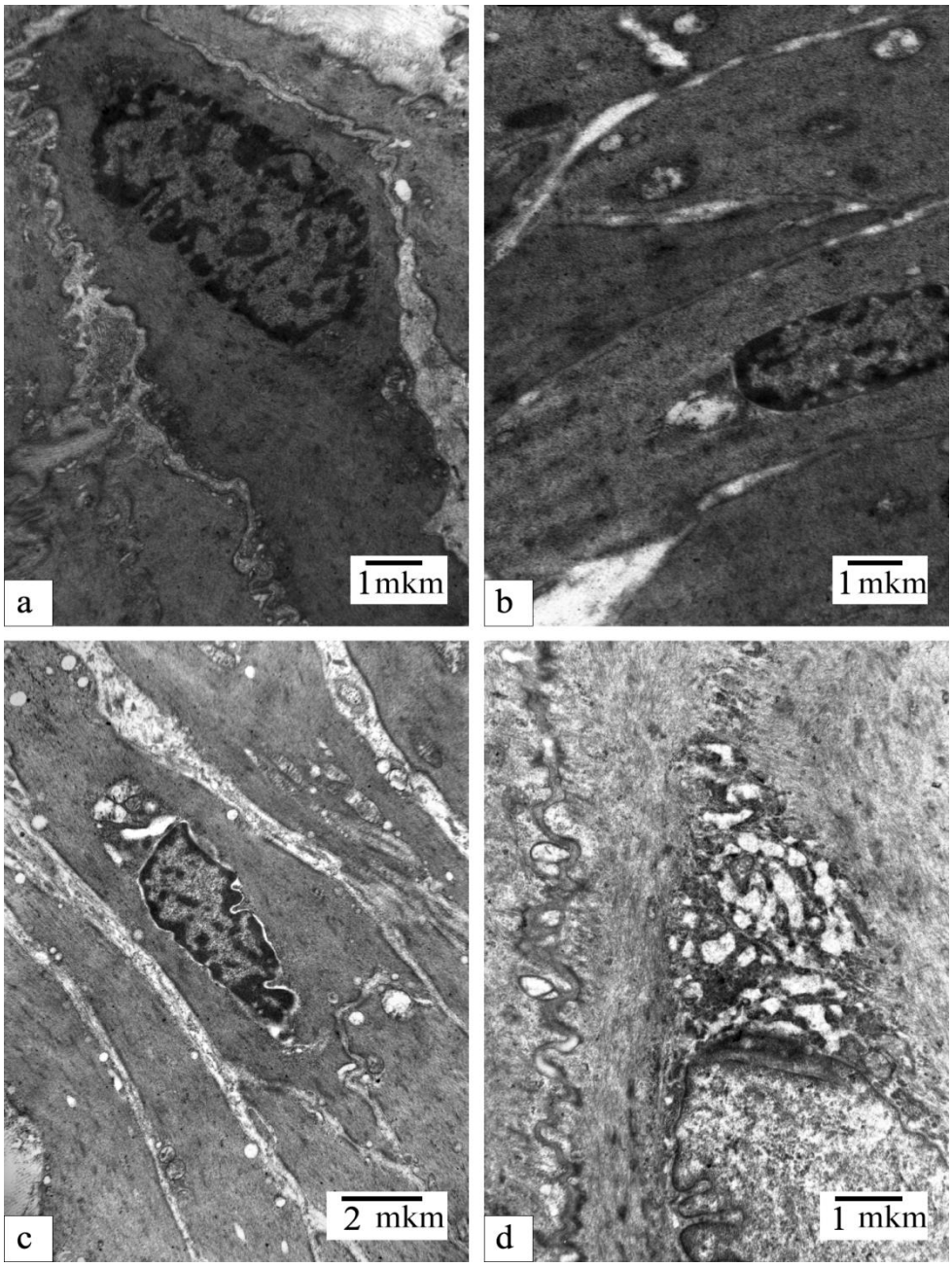

Fig. 3. Ultrastructure of the dark functioning smooth myocytes (a) and dark myocytes of the involutive type (b) and smooth myocytes with signs of vacuolar dystrophy (c, d) during the 14^{th} (a), 28^{th} (c, d) and 42^{nd} (b) day of the course of diabetes mellitus. Transmission: electron microscope images.

We believe that the cause of vacuolar degeneration of the UB smooth myocytes, which is already from the 14^{th} day of DM, during the 42^{nd} day is supplemented by baloon dystrophy and can be traced up to the 56^{th} day, there is urine, which is hypotonic due to the repeated increase in diuresis speed resulting of glomerular hyperfiltration [4]. We have determined [35] that the daily urine output in diabetic rats during the 14^{th}, 28^{th}, 42^{nd} and 56^{th} day of the experiment is increased compared with the control group respectively 10.9, 15.8, 10.8 and 7.7 times ($p<0.001$ – 0.01). In specified periods there is also desquamation of transitional epithelial cells that leads to its atrophy and destruction of urothelial cell barrier. Under these conditions, as indicated by a number of

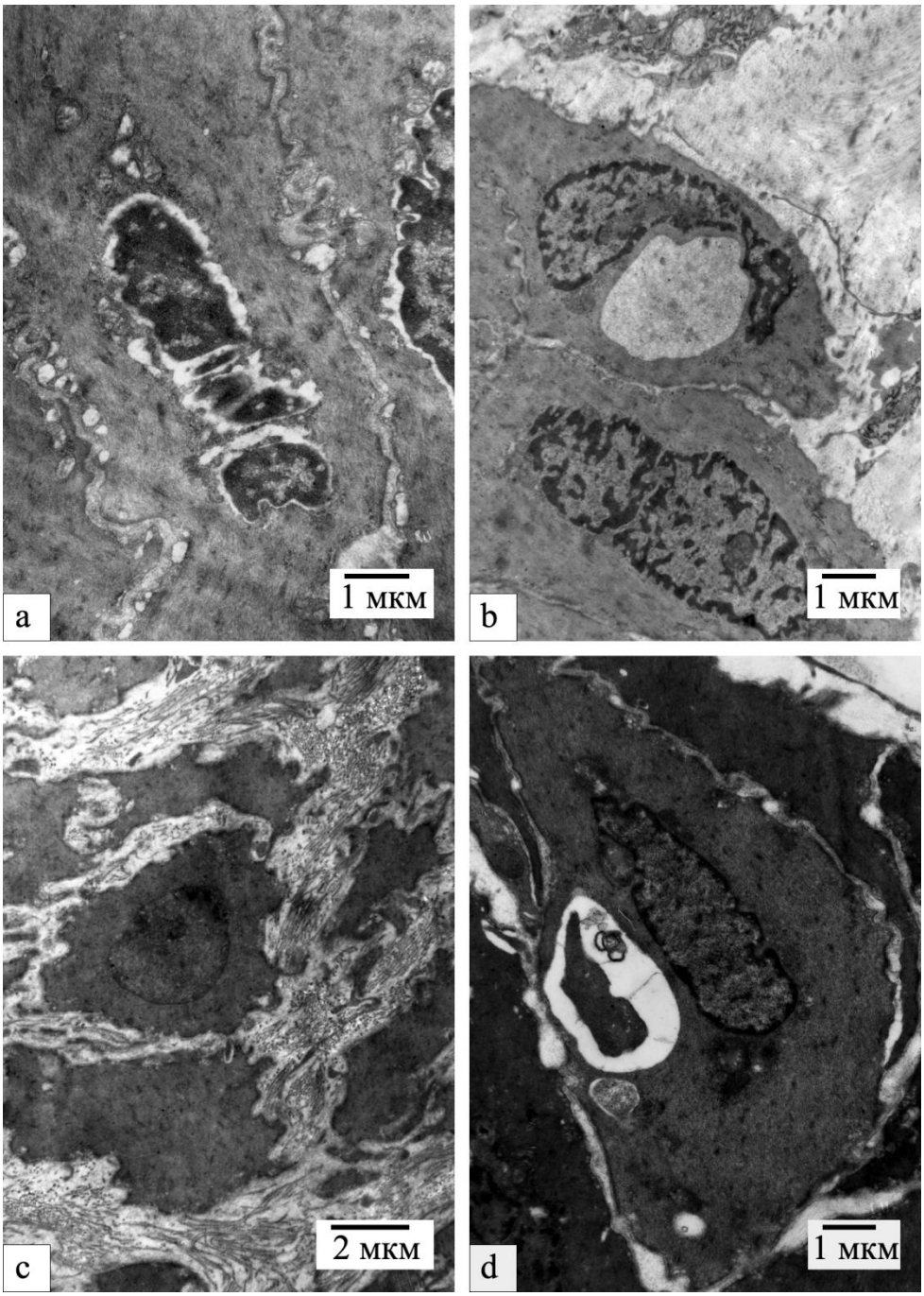

Fig. 4. Electronic microscopy of UB wall during the 42nd day of DM development: smooth myocytes with vacuolar (a) and baloon (b) dystrophy. 70th day of observation: areas with focal fibrosis (c) and sequestration of smooth myocytes (d). Transmission: electron microscope images.

authors [3, 16], there is advancement of urine into the deeper UB layers, in this case – hypotonic urine, which, as it is noted by G. Apodoca [1], causes swelling of the cells. However, there is the report of experimental research in which transurethral single acute hyperextension of UB was performed [9]. The authors have determined that after this edema occurs in its own plastic and between smooth muscular fibers, and myocytes have a significant number of vacuoles. We can neither confirm nor refute these results, but assume that such a mechanism of water-mineral metabolism violation in the muscular tissue of UB in

DM is quite possible, because in this pathology rapid hyperextension of the UB as a result of polyuria occurs constantly. In addition, some authors suggest that in benign prostatic hypertrophy vacuoles of various size are present in the cytoplasm of almost all UB smooth myocytes [20, 23].

Through all the terms of DM development, we have observed polymorphism of ultrastructural restructuring of the UB smooth myocytes of diabetic rats. The following changes of the UB myocytes in hyperactive UB and prostatic hyperplasia are indicated by a number of Russian authors [20, 23], who consider these changes as stereotypical options of structural modification of muscular layer of UB in various pathological processes and call them phenotypical heterogeneity.

4. Conclusions

1. The early period of DM development (14^{th} – 28^{th} day) is characterized by a decrease in percent of collagenous fibers area and increase of the percentage of smooth myocytes area in the wall of UB. Ultrastructurally there is expanding of intercellular and perinuclear spaces and development of vacuolar degeneration of myocytes, manifestations of which are different. During the 42^{nd} – 56^{th} day the percentage of collagenous fibers area increases and the percentage of the smooth myocytes area decreases. Ultrastructurally further development of vacuolar dystrophy, signs of which subside by the end of the period, short-term and moderate baloon dystrophy, appearance of the dark myocytes poor in organelles, moderate karyorrhexis are observed. During the 70^{th} day of the experiment percent of collagenous fibers and smooth myocytes areas are not significantly different from the previous period; most dark myocytes are of involutive type, there are areas of local fibrosis and myocyte sequestration.

2. Important peculiarities of diabetic cystopathy that is streptozotocin-induced, are pronounced polymorphism of ultrastructural changes of smooth myocytes, which has a chronological dependence, and also the fact that during the 70 days of DM the diabetic dysfunction of the UB in rats is compensated.

3. Hypertrophy of smooth myocytes and the relative decrease of the area of collagenous fibers should be considered as interrelated compensatory responses to repeatedly increased diuresis that provide the increased urinary ability of the UB and simultaneously increase susceptibility of its walls to stretch. However, these processes develop asynchronously and disproportionately to changes in urine volume, indicating the existence of additional factors that influence their course.

4. Our worked out method of analysis of the structural components of the UB wall in ImageJ allows to determine the percent of smooth muscle and collagenous fibers areas in the automatic mode and excluding the area occupied by the main substance of the extracellular matrix. This leads to entirely new opportunities for morphometric analysis of UB walls, which are expressed in the elimination of the subjective factor, levelling of individual variability of animals and the results that are highly variable at different stages of bladder wall stretching and various states of the basic substance of the extracellular matrix.

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