Morphology of the submandibular gland's acini of rats in diabetes mellitus

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Aim. The purpose of the study was to determine the morphological changes in seromucous acini of the submandibular gland in experimental rats with diabetes mellitus.

Material and methods. The experiment was performed with 1-year-old male Wistar rats. Experimental diabetes mellitus was induced by a single intraperitoneal administration of streptozotocin. Ultrastructural changes in the seromucous cells of the submandibular gland were studied during the 14th, 42nd and 70th days of the experiment.

Results. In experimental diabetes mellitus in the seromucous cells of the submandibular gland hypertrophy of the rough endoplasmic reticulum and the Golgi complex; vacuolization of the cytoplasm; a large number of intracellular lamellated structures and lysosomes; diffuse homogenization of the matrix, destruction of mitochondrial cristae and vacuolization of mitochondria were observed and the most pronounced on the 14th day. On the 70th day of the experiment the seromucous cells are characterized by the absence of morphological signs of damage and the development of atrophy and it is confirmed by the reduction in cells' size as well as the normalization of the relative area of secretory granules.

Conclusion. The obtained data indicate that the reaction of the cells of seromucous acini to the development of hyperglycemia is typical and nonspecific. Thus, the alternative changes have a pronounced dystrophic character in the early stages and increase to the 42nd day of the experiment. On the 70th day histologic adaptation of the seromucous acini was observed and approved by atrophic changes, decrease of cells' area and normalization of the relative area of their granules.
A number of studies has found that there is a correlation between diabetes mellitus (DM) and dental pathology including the dysfunction of the salivary glands [1–4]. Diagnostic of salivary gland dysfunction is rather difficult in clinical practice and based on systematic patient examination [5]. The submandibular gland (SMG) is the main source of basal salivary secretion [6,7]; hence, the abnormalities in the functioning of its seromucous acini play the leading role in the development of hyposalivation and xerostomia [8]. Such correlation between the salivary glands and DM confirms a clear association between hyperglycemia and oral health status, thereby indicating the relevance of studying SMG.

It should be noticed, main function of acinar cells is primary saliva production. Its base is secretory cycle and consists of receiving substances, synthesis, storing and eliminating of secretory granules. During secretory cycle, volume of cells and their secretory granules changes as main volume of primary saliva is accumulated and then is excreted under different stimuli such as food, autonomic nerve stimulation, etc. [9,10].

A lot of researches highlight changes of the acini of SMG, which are seromucous in rats [11], under influence of DM [11–16]. At the same time, structural normalization of the seromucous cells of SMG (except hypertrophy of the rough endoplasmic reticulum) was indicated in Wistar rats after 3 weeks of hyperglycemia [12].

According to [13], volume of the acini of SMG did not undergo any changes after 4 weeks after streptozotocin-induced diabetes. However, on electron microscopic level, autophagosomes and lysosomes were often detected [14].

Impact of streptozotocin-induced hyperglycemia during 6–7 weeks leads to salivation decrease almost twofold and reducing the activity of salivary amylase more than fivefold [15]. Also, dystrophic changes of SMG acini was found [16] after 8 weeks of experimental DM. However, structural normalization of the seromucous cells and the reduction of their size were observed over a 2-month period of hyperglycemia [12].

Summarizing all the aforementioned information, it can be affirmed that experimental DM is accompanied by disturbances in the metabolism and antioxidant status of the seromucous cells. The results of morphological studies [11–16] are often controversial; although, they indicate the development of dystrophic changes, but differ in experimental design, lines of rats and their initial age, the duration of hyperglycemia exposure. All these factors lead to the difficulties when comparing the results of the features of morphological changes of the seromucous acini in experimental hyperglycemia.

**Aim**

The objective of research was to determine chronological dependence between morphological changes in seromucous acini of SMG and duration of the experimental DM.

**Materials and methods**

The study included 1-year-old male Wistar rats which were divided into 2 groups: the experimental (n = 15 animals) and the control (n = 15 animals). DM was induced by an intraperitoneal administration of streptozotocin (60 mg/kg body weight) dissolved in cold citrate buffer pH 4.5. Animals of the control group were injected with an equivalent amount of citrate buffer only. The development of DM was proved by monitoring of glucose level in the blood (fasting blood samples was collected from tail vein and measured by glucometer Accu-Check Active, Roche Diagnostics GmbH, every day during experiment). Animals, having fasting blood glucose concentration over 12 mmol/l were considered as diabetic ones.

The samples were collected in the morning, before food intake, on the 14th, 42nd and 70th days after an overdose of thiopental (5 animals per group).

The left SMG was excised and fixed in 10 % buffered paraformaldehyde solution (pH = 7.4), embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin, examined with light microscope Micros Austria MC300 and photographed with ToupCam 5.1M UHCCD C-Mount Sony.

The right SMG was removed and fixed in 2.5 % glutaraldehyde solution with followed post-fixation in 2 % osmium tetroxide (OsO₄) solution for electron microscope examination. Electron microscopy was performed with PEM-125K.

Morphometry was conducted in ImageJ2 [17], as it allows to perform image analysis in semi-automatically mode using build-in macro language [18]. On histological sections the area of the seromucous cells was measured. On transmission electron micrographs of the seromucous cells, the RA of their secretory granules was determined by morphometric analysis. The main point of the developed algorithm was as follows:

1. The contours of the seromucous cell were outlined (manually) using the command “Fit Spline” (Fig. 1a);
2. The area outside the current image selection was erased to the background color (the command “Clear Outside”). Using the command “Threshold” (method “IJ_Isodata dark”, the maximum threshold value was 254; the minimum threshold value was automatically adjusted) secretory granules were covered by the mask (Fig. 1b);
3. Outliers were removed (the command “Remove Outliers”), there were used binary operator Close (iteration = 5 & count = 3) and the “Watershed” and “Fill Holes” commands (Fig. 1c);

4. “Analyze Particles” module was used to determine the area of particles with a circularity of 0.5 to 1.0 (determined empirically) (Fig. 1d).

Basing on [7,9,10], such morphometric parameters have been chosen as indicators of the secretory activity of the cells.

Statistical analysis was conducted using R software [19]. Results of the descriptive statistics are shown as Mean ± SD. The data of independent groups were compared using the Mann–Whitney–Wilcoxon test; the difference was considered statistically significant at $P < 0.05$.

Ethical approval. The experiment was carried out in accordance with EU Directive 2010/63/EU for animal experiments.

Results

The 14th day after streptozotocin administration. On the background of hyperglycemia (blood glucose level was $14.02 \pm 1.20$ mmol/l exceeded that in the control group 2.74-fold, $P < 0.01$), the seromucous cells with large vacuoles were seen (Fig. 2a). There were round nuclei with small invaginations of the nuclear membrane and predominant euchromatin; heterochromatin seen in the form of clumps was mainly marginally distributed; the perinuclear space was identified around the perimeter; the extended perinuclear space was occasionally seen. The seromucous cells with hypertrophied rough endoplasmic reticulum (RER) and Golgi complex (GC) were prevalently observed (Fig. 2b). The cisternae of the GC were often dilated. Mostly elongated mitochondria of different sizes with unclear cristae were found at the cell periphery. In single cells, autolysosomes and intracellular laminated structures were detected. The areas containing disorganized, vacuolated, dilated cisternae of the RER and a lower number of ribosomes attached to their surfaces were occasionally seen.

It was morphometrically determined that during this observation period the area of the seromucous cells did not differ from that in the control group ($P > 0.05$, Table 1). The RA of secretory granules reduced 1.42-fold compared to control values ($P < 0.001$).

The 42nd day of experiment. The seromucous cells containing a low number of secretory granules were identified (Fig. 3a), their RA was 1.37 times lower than control values ($P < 0.01$). The cisternae of the RER and GC were dilated. Mitochondria were mostly rounded; the destruction of their cristae and homogenization of
the mitochondrial content were observed. The perinuclear space was not clearly identified around the perimeter of the nucleus; the vesicles transporting lysosomal enzymes, heterolysosomes and autolysosomes were seen (Fig. 3a,b).

On the 42nd day, on the background of the increase in blood glucose level (23.88 ± 3.16 mmol/l) which exceeded that in the control group 4.68-fold (P < 0.001), the area of the seromucous cells reduced 1.15-fold (P < 0.001) compared to the control group (Table 1).

The 70th day after streptozotocin administration. Numerous areas of the overgrowth of glandular stroma were seen (Fig. 4a). The seromucous acini were formed by small seromucous cells, the nuclei of which contained mostly euchromatin; heterochromatin was located under the nuclear membrane. The perinuclear space was preserved; the extended perinuclear space was occasionally observed. Only few cisternae of the RER and GC were seen (Fig. 4b). Mitochondria of different sizes and shapes with clear cristae and the enlightened matrix were found at the cell periphery. The vesicles transporting lysosomal enzymes were single; secretory granules were enlightened.

Despite stable level of blood glucose (22.93 ± 2.28 mmol/l) as compared to the 42nd day (P > 0.05), a progressive reduction in the area of the seromucous cells 1.67-fold (P < 0.001) was observed (Table 1), while the RA of secretory granules rapidly increased and did not differ from control values on the 70th day of the experiment (P > 0.05).

Table 1. Area of the seromucous cells (μm²) and relative area of their secretory granules in the rat submandibular gland during different time periods (Mean ± SD)

| Time period, day | Animals   | Area of seromucous cells, μm² | Relative area of granules |
|------------------|-----------|-------------------------------|---------------------------|
| 14               | Control   | 120.23 ± 22.82                | 0.44 ± 0.05               |
|                  | Experimental | 124.62 ± 34.36            | 0.31 ± 0.12**             |
| 42               | Control   | 123.55 ± 23.56                | 0.48 ± 0.05               |
|                  | Experimental | 107.47 ± 25.41**          | 0.35 ± 0.16*              |
| 70               | Control   | 115.79 ± 21.36                | 0.45 ± 0.04               |
|                  | Experimental | 69.44 ± 16.58**            | 0.42 ± 0.09               |

*, **: the level of statistical significance achieved in comparison with the control group, P < 0.01, P < 0.001, respectively.
Discussion

In streptozotocin-induced diabetes, on the background of dynamic increase in the levels of blood glucose, pronounced dystrophic changes in seromucous cells being characterized by vacuolization of the cytoplasm, a large number of lysosomes, intracellular laminated structures, mitochondria with vacuolated content and crista destruction were observed during the early observation periods (on the 14th–42nd days of the experiment). Moreover, hypertrophy of the RER and GC cisternae were present. Such morphological changes indicate changes in the cells’ condition and, probably indicates the activation of compensatory mechanisms, cells’ swelling and damage, and, as a result, the abnormalities in the processes of secretion [20,21]. Similar changes in the seromucous cells of SMG in streptozotocin-induced diabetes were observed by Mednieks et al. [14]. At the same time, 3 weeks after streptozotocin administration, most seromucous cells are characterized by normal ultrastructure without significant pathological changes, except for the increase in the amount of the RER [12]. In our opinion, such differences occurred due to the fact that we have used 1-year-old male rats, because initial age has an important effect on experimental results [22]. The dynamics of morphometric parameters deserves special attention. On the 14th day, the RA of secretory granules reduced while there were no statistically significant changes in the sizes of cells. In our opinion, it indirectly point out a potential predominance of atrophic mechanisms, cells’ swelling and damage, and, as a result, the abnormalities in the processes of secretion [20,21]. The obtained data indicate that the reaction of se- romucous cells to the development of hyperglycemia is typical and nonspecific. Thus, the alteration changes have a pronounced dystrophic character in the early stages and increase to the 42nd day of the experiment. On the 70th day histologic adaptation of the seromucous acini was observed and approved by atrophic changes, decreasing area of cells and normalization of the relative area of their granules.

Conclusions

The obtained data indicate that the reaction of seromucous cells to the development of hyperglycemia is typical and nonspecific. Thus, the alteration changes have a pronounced dystrophic character in the early stages and increase to the 42nd day of the experiment. On the 70th day histologic adaptation of the seromucous acini was observed and approved by atrophic changes, decreasing area of cells and normalization of the relative area of their granules.

Conflicts of interest: authors have no conflict of interest to declare.
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