Morphological and functional features of large salivary glands of rats against the background of prolonged alcohol intoxication of the body

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Abstract. Using classical methods of morphometric and histochemical analyzes, changes in the morphological structures and enzymatic activity of the submandibular (SMG) and parotid (PG) salivary glands of male Wistar rats were revealed against the background of long-term drinking of 20% ethanol solution for 180 days. The most pronounced changes were found in the SMG.

Keywords: parotid salivary gland, submandibular salivary gland, ethanol, mast cells, alkaline phosphatase, succinate dehydrogenase, NADP oxidase

Homeostasis of the oral cavity is determined by many factors, but, first of all, by the functional activity of the salivary glands, which contribute to both the presence of dental pathology and the somatic health of a person as a whole. In many countries of the world, excessive alcohol consumption negatively affects the health of the population. The effect of ethanol can be carried out directly on the mucous membrane of the oral cavity, which is characterized by a high degree of permeability, and also indirectly, through the products of ethanol metabolism after a number of transformations in the body and their excretion with saliva. The negative effects of ethanol on the morpho-functional state of the salivary glands are obvious [1-5]. However, most of the studies were completed in a short time. For an objective analysis, it is necessary to conduct a comprehensive study of changes in the morphological structures and enzymatic activity of SMG and PG rats with prolonged alcohol intoxication.

Materials and methods. The study was carried out on adult male albino rats of the Wistar line weighing from 180 to 210 g. The animals were divided into 2 groups. Group 1 - control (n=20), group 2 - experimental (n=20). Experimental animals received a 20% solution of ethyl alcohol (7 g/kg/day) as a source of drinking for 180 days. All animals were kept in standard

vivarium conditions: with standard temperature conditions, 12-hour light/dark cycle, with free access to food and water (GOST 33215–2014 RF entered into force on 01.07.2016).

The selection, observation of laboratory animals was carried out according to the recommendations of I.M. Trachtenberg [6]. All actions involving contact with laboratory animals were carried out taking into account the rules for working with experimental animals [7] and the Federal Law of the Russian Federation "On the Protection of Animals from Cruelty" dated 01.12.1999 № 4679-II GD [8]. To conduct this study, permission was obtained from the University Ethics Committee (protocol № 3 of November 16, 2015) in accordance with the order of the USSR Ministry of Health № 775 of August 12, 1977 [7].

Salivary gland collection

The animals were anesthetized with ether at the rate of 3-5 ml per 1 kg of body weight mixed with atmospheric air. After the absence of the corneal reflex, the rats were decapitated. Then, large SMG and PG were collected, which were subjected to morphological and histochemical research methods.

General morphological analysis

Material SMG and PG were fixed in 10% neutral formalin, then dehydrated in alcohols of ascending strength and embedded in paraffin according to the standard technique. Sections of salivary glands 5-7 μm in size, made using an MPS-2 microtome (Kharkov, Ukraine), were stained with hematoxylin-eosin. Sections were obtained for morphometric analysis taking into account the area of the parenchyma, stroma, acini and ducts in accordance with the protocol of Merlo C. et al. 2010 [9]. Description, comparative morphological analysis of SMG and PG structures was carried out on permanent microslides using a microscope (Mikmed-2, Russia) at magnifications of 10x10, 10x40 and 10x90.

Characterization of mast cells

To qualitatively and quantitatively characterize the population of mast cells (MC), we used the method of staining with polychrome toluidine blue according to A. Unna [10]. In 30-50 fields of view of a light microscope at a magnification of 10x40 in each section, the number of MCs in SMG and PG was counted, and the arithmetic mean values were found for each case. The sizes of MC were determined after photographing the preparations at a magnification of 10x90 using the "SigmaScanPro 5.0" software package. Each MC was characterized by the degree of degranulation. The degranulation index (DI) was calculated using the formula: DI = C/B, where C is the number of degranulating MCs, B is the total number of analyzed MCs. In addition, the degree of metachromasia, visualization of granules and nuclei were determined [11]. According to the degree of metachromasia, α-orthochromic MCs (blue, contain non-sulfated heparin), β-

metachromatic (purple, heparin sulfated to varying degrees), γ-metachromatic (purple, containing highly sulfated heparin) were isolated.

Histochemical analysis of enzyme activity. Alkaline Phosphatase Activity

Method for determination of alkaline phosphatase (AP) activity in acini and SMG and PG ducts according to Burstone M.S. based on the hydrolysis of monosubstituted orthophosphate esters [12]. The reaction proceeds with the simultaneous azo coupling of the substrate - naphthol-AS-BI-phosphate (Sigma-Aldrich, USA) with a dye - strong blue BB (Sigma-Aldrich, USA). Alkaline phosphatase breaks down α-naphthol-phosphate with the release of α-naphthol. In the places of localization of the enzyme, an insoluble blue precipitate is formed.

To determine the AP activity, freshly prepared cryostat native sections of salivary glands 10 μm thick were dried. The sections were then incubated with naphthol-AS-BI-phosphate and strong blue BB for 20 min at + 37°C. An alkaline Tris HCl buffer with pH = 8.2-9.2 (Jiangsu Juming Chemical Technology Co., Ltd., China) was used as the basis of the incubation medium. AP activity was quantified using cytophotometry. Photometry was carried out in transmitted light on a microscope (Mikmed-2, Russia) using an FMEL-1 photoelectric attachment with FEU-79 (Russia) and an amplifier output voltage of 900 V. In order to obtain a monochromatic light beam in the red region of the spectrum passing through the preparation, an interference light filter (Russia) with a maximum light transmission (λmax = 620 nm) was used. Light transmission was recorded using a digital voltmeter. Then, by negative logarithm, the level of light transmission was transformed into light absorption, and the optical density was calculated. According to the Lambert-Bouguer-Beer law, the optical density of a drug is proportional to the amount of dye. In turn, the described method meets the requirement of proportionality of the concentration of the dye and the activity of the enzyme.

Activity of succinate dehydrogenase and NADPH-oxidase

In freshly prepared cryostat sections, the content of succinate dehydrogenase (SDH) and NADPH-oxidase was determined by the method of Z. Lojda [13]. For this, the native sections of the salivary glands with a thickness of 10 μm were dried and then incubated in a tetrazolium reagent for 15 min. The reaction is based on the transformation of the colorless oxidized form of tetrazole salts into a colored reduced form. Enzymatic activity was determined quantitatively using photometry.

The results of the study were subjected to *statistical analysis* using the "*BioStat 2009 Professional 5.8.4*" software (*AnalystSoft*, USA). To assess the differences, the nonparametric Mann – Whitney U-test was used, considering the differences to be significant at $p<0,01$. The results of the study are presented in the table and in the text as the median and the 25th and 75th centiles (*Me, Q_1-Q3*).

3. Results

The body weight of the animals during 180 days did not have statistically significant differences between the groups. During the entire period of the protocol of alcohol consumption, no animal deaths were recorded.

Morphological analysis

When examining sections of PG and SMG animals of the control group, we showed that the structural organization of each organ corresponded to the norm.

In experimental animals exposed to chronic alcohol intoxication (180 days), no significant violations of the general structural organization of the terminal secretory sections and excretory ducts of PG were found. However, changes in the shape of the end sections and variability in the size of the secretory cells that form them were often noted, among which there were also very large ones. In the cytoplasm of many cells, round, unstained vacuoles were observed, which presumably correspond to fatty inclusions. The stroma had moderate fatty infiltration in the form of large adipocytes with isolated lymphocytic foci. Blood stagnation was observed in the blood vessels. The interlobular excretory ducts are unevenly expanded, their cells were of different heights.

In the SMG of the experimental group of animals, mild metachromasia with blue toluidine was observed in all the studied acinar cells of the glands. In some acinar cells, nuclei of irregular shape were found, with varying degrees of heterochromatin inclusions. Lipid inclusions located in different areas of the cytoplasm were observed in almost all cells of the acinus. In some lobules, small atrophic acini were found. The striated ducts showed alternation between dark and clear cells, with atypical, heterochromatic nuclei, irregular contour and located at different levels. The cells of the intercalated ducts contained some lipid droplets. The duct epithelium had nuclear heterogeneity and moderate atrophy with accumulation of secretory material. Fatty infiltration and stromal edema were observed.

On sections PG and SMG of the control group, β-metachromatic MCs with a size of about 10-25 μm in an amount of 4.5 c.u. in the field of view were clearly detected. MCs are mainly concentrated in groups near the interlobular ducts and blood vessels. The identified MCs are predominantly without signs of degranulation, oval or elongated, with well-defined margins and cytoplasm filled with densely spaced metachromatic granules. There were also cells with signs of partial degranulation, without clear boundaries, different sizes, and irregular shapes. The cytoplasm of such MCs is less intensely colored due to loosely located, well-distinguishable granules. Outside of these cells, a large number of granules were contained. The degranulation index was 0.3.

The number of identified MC PG and SMG experimental groups increased to 8.5 c.u. in the field of view. In the connective tissue septum near the interlobular ducts, MC formed conglomerates. The content of MCs, located inside the lobules, near the terminal sections, intercalated and striated excretory ducts, slightly increased. Whole MCs were single, the main part of the cells was in the stage of degranulation, respectively, the index of degranudation was 0.6. Moreover, all of them were characterized by a high degree of activity - enhanced maturation and release of MC granules. An increase in the number of MCs degranulating by total cytoplasm disintegration was observed. Completely destroyed MCs with light cytoplasm and a small number of scattered, metachromatically stained granules made up 1/3 of the number of degranulated ones.

AP activity

It was shown that in animals with alcohol intoxication after 180 days, in comparison with the control, there was a statistically significant increase in AP activity in serocytes of terminal acini SMG and PG (table 1). At the same time, in the striated ducts of the salivary glands, the level of enzyme activity did not differ between the groups.

Table 1

Alkaline phosphatase activity in the PG and SMG structures during alcohol intoxication for 180 days (ME; Q1-Q3)

Structure	Gland	Series	
		control	experimental
acini	PG	0.45 (0.395 – 0.69)	0.86 (0.765 – 0.99) ($p<0.01$)
	SMG	0.43 (0.40 - 0.52)	0.87 (0.84 - 1.095) ($p<0.01$)
ducts	PG	0.44 (0.40 - 0.49)	0.48 (0.40 - 0.54)
	SMG	0.44 (0.39 - 0.52)	0.47 (0.40 - 0.51)

SDH and NADPH-oxidase activity

We found that the SDH activity in the ducts and acini of the salivary glands did not statistically differ between the control and experimental groups of animals (table 2).

The results of the studies showed (table 2) that with prolonged 180-day intoxication with ethanol in the acini and ducts of PG rats, the activity of NADPH-oxidase remained at the control level. However, in the SMG acini of the experimental group, the enzyme activity increased statistically significantly, while in the ducts it remained unchanged in all groups.

Table 2

Activity of SDH and NADPH-oxidase in the PG and SMG structures during alcohol intoxication for 180 days (IU; Q1-Q3)

Structure	Gland	Series			
		control		experimental	
		SDH	NADPH-oxidase	SDH	NADPH-oxidase
acini	PG	69 (64.5 – 72)	76 (69 – 86)	63.5 (59 - 76)	77 (70.25 - 81)
	SMG	58 (53.5 - 61)	77 (74 - 81)	54 (42 - 69)	82 (78 - 85) ($p<0.01$)
ducts	PG	40.5 (34 - 45)	62 (52 – 68.75)	38 (27.25 - 45)	55 (47.75 – 69.25)
	SMG	25 (19.25 - 34)	69.5 (66 - 77)	19 (16 - 44)	63 (55 - 65)

Discussion

Our results showed that long-term ethanol consumption (180 days) in an amount of 7 g/kg per day causes different morphological changes in PG and SMG. More negative impact has affected SMG. If in PG the stroma had moderate fatty infiltration in the form of large adipocytes with the presence of isolated lymphocytic foci, then in SMG complete fatty infiltration and stromal edema were observed. The lipid inclusions found in the acinar cells of the salivary glands are probably associated with disorders of fatty acid metabolism and autonomic innervation of the glands [14]. In addition, more significant structural changes in the acini and ducts were noted in the SMG, which is probably a way to repair the damage caused by alcohol intoxication. It is generally accepted that PG is more vulnerable to toxic agents under the condition of short-term alcohol intoxication [15, 16]; our study covered a longer period of time - 180 days. Probably, PG cells gradually adapted to chronic ethanol intoxication, while SMG cells are more resistant to short-term exposure to the toxin.

We paid special attention to the study of MC, since their state in PG and SMG under conditions of chronic alcohol intoxication of the body remains poorly understood. We have shown that under conditions of chronic alcohol intoxication there was a clear increase in the number of MCs in the salivary glands, especially in the connective tissue layers near the excretory ducts, which is consistent with the literature data, which indicate an increase in MC content in various organs under the influence of alcohol [17]. At the same time, it is interesting that the absolute majority of MCs were in a state of degranulation, due to which the degranulation index doubled. The salivary glands are the organs that excrete ethanol and its metabolite acetaldehyde. Thus, we have shown that, entering the salivary glands, ethanol as an irritant causes a high degree of MC activation, accompanied by their enhanced maturation and intense degranulation. It can be assumed that such a high degree of MC degranulation under the influence of alcohol intoxication causes activation of additional regulatory mechanisms in the

salivary glands aimed at accelerated excretion of ethanol and toxic metabolic products from the body.

It is known that the toxic effect of alcohol on the body changes the activity of alkaline phosphatase. It was shown that short-term (10 days) ethanol intoxication of animals did not affect the enzyme activity in the intestine; however, it markedly increased after 20 or 30 days, and after 42 days it decreased both in the soluble and in the membrane fractions of the intestine [18]. Dal Prá, K.J. et.al. have shown that 30-day consumption of the strong Brazilian drink cachaça causes a decrease or complete loss of the functional activity of alkaline phosphatase in the SMG [19]. Our data indicate a significant twofold increase in the enzyme activity in SMG and PG rats after 180-day ethanol intoxication. Probably, this fact may indicate the degree of disturbances in physiological processes in the body.

The early manifestations of alcoholic pathology are based on a violation of tissue respiration due to a change in the level of components of the respiratory chain and an increase in the production of free radicals. SDH plays not only a central role in the Krebs cycle and the respiratory chain, but also differs from other mitochondrial dehydrogenases in its unique redox effect. We have shown that under conditions of 180-day intoxication, the level of SDH activity in the salivary glands of rats remains the same as in the control group. Therefore, this enzyme is quite stable.

It is known that ethanol activates NADPH-oxidase [20]. We found that after prolonged 180-day intoxication with ethanol in the acini and ducts of PG rats, the activity of NADPH-oxidase remained at the control level, while in SMG only in the acini the activity of the enzyme increased statistically significantly. Therefore, by our research, we confirmed that PG showed greater resistance to prolonged alcohol intoxication, while SMG is more susceptible to oxidative stress and associated morphological changes.

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