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Cytopathological characteristics for provoked lesions of squamous epithelia of the digestive system in animal models

Relevance: Noticeable spreading of malignant neoplasms of the upper section of the digestive system and the worsening of these patients' quality of life in case of late diagnostics stimulate the development of protocols for early detection of precancerous changes.

The purpose of the study was to investigate the morphological characteristics of changes in the squamous epithelium of the upper part of the digestive tube in model organisms under provoked carcinogenesis when exposed to substances with proven carcinogenic activity.

Methods: 40 female nonlinear white rats from the Orekhovo-Zuevsky urban settlement veterinary station were used as model organisms. The rats were divided into four equal groups. The first control group was not exposed to any specific influences. In groups 2-4, the rats underwent sedation and mechanical scarification microtraumatization of the oral mucosa. Then, they were applied twice a week by 0.9% NaCl solution (control group 2), 1% aqueous solution of dimethyl sulfoxide (DMSO, Akrikhin, Russia) (control group 3), or 1% aqueous solution of DMSO containing 0.1 mg/ml 4-nitroquinoline-N-oxide (4-NQO, Acros Organics, USA) (Experimental Group 4).

Results: The cytological picture associated with chronic trauma to the mucous membrane of the upper digestive tract was characterized by nonspecific reactions: inflammation, hyper- and parakeratosis, and atrophic changes. There were no statistically significant differences in the frequency of such changes between the experimental and control groups. Specific changes in squamous epithelium towards a precancerous state in the Experimental Group with chronic exposure to a substance with carcinogenic activity occurred significantly more often than in the control groups ($p < 0.05$), as proven by the results of comparative histological verification.

Conclusion: Cytological study could be used in clinical practice as a screening tool for precancerous oropharynx and upper esophagus conditions.

Keywords: malignant tumors, cytology, intraepithelial lesions, squamous dysplasia, digestive system, animal models.

Introduction: Esophageal cancer is one of the most aggressive malignant neoplasms. According to the International Agency for Research on Cancer (IARC), 572,000 new cases of esophageal cancer were registered in the world in 2018 (3.2% of all malignant neoplasms) and 508,600 thousand deaths from esophageal cancer (5.3% of all deaths from cancer, 6th ranking place) [1]. The search and study of common morphological and immunological markers of malignancy, as well as the diagnostic precursors of invasion and metastasis, are inherently associated with the development of reliable diagnostic protocols for early diagnosis of tumor diseases of the esophageal mucosa [2].

Despite the recommendations for taking the primary diagnostic material during the patient's first visit, there are no uniform approaches to preparing the material, its transportation, and pre-analytical processing [3]. At the same time, the cytological material can provide diagnostically sig-

nificant morphological data. The simplest methods of preparing the research material, such as staining by Romanovsky-Giemsa, Pappenheim, Papanikolaou, etc., are potentially available to all laboratories of anatomic pathology departments [4]. The cytological material can also be suitable for the ultrastructural analysis and study of the immunological profile [5]. The pilot studies have shown that the Bethesda diagnostic criteria - a universal system for interpretation and evaluation of the cytological studies' findings, can be used in the case of oral cavity carcinoma [6, 7]. However, the lack of a unified classification system for changes detected by a doctor in cytological smears significantly limits the possibilities for early diagnostics with the minimally invasive methods. The number of national authors highlighted the essential details of the cytopathological picture associated with metaplasia of the esophagus squamous epithelium [5]. Further studies in this area will provide an idea of

the common properties of the altered squamous epithelium cells.

The purpose of the study was to investigate the morphological characteristics of changes in the squamous epithelium of the upper part of the digestive tube in model organisms under provoked carcinogenesis when exposed to substances with proven carcinogenic activity.

Materials and Methods: 40 female nonlinear white rats obtained from the Orekhovo-Zuevsky urban settlement veterinary station were used as model organisms (Veterinary certificate No. 8431768363 as of 25.01.2021). The animals were six weeks old at the start of the experiment. The animals were kept in the university vivarium for a 14-day quarantine before the test. The rats were divided into four equal groups ($n = 10$ each). The first control group was not exposed to any specific influences and was considered a reference for this study. In groups 2-4, the rats underwent ether sedation and mechanical scarification microtraumatization of the oral mucosa. Then, they were applied twice a week by 0.9% NaCl solution (control group 2), 1% aqueous solution of dimethyl sulfoxide (DMSO, Akrikhin, Russia) (control group 3), or 1% aqueous solution of DMSO containing 0.1 mg/ml 4-nitroquinoline-N-oxide (4-NQO, Acros Organics, USA) (Experimental Group 4). The animals were kept under the standard vivarium conditions with free access to food and water. Feeding was carried out with compound feed for laboratory animals. Potable water was used for the drinking of animals according to SanPiN 2.1.3684-21.

There is evidence of the predictive value of non-neoplastic conditions of the oral mucosa, detected during endoscopic examination and elevation of risk for the esophagus squamous cell carcinoma [8]. In this regard, within the frames of this study, the morphological predictors of local dysplasia in the epithelium of the oral cavity were taken as prognostic and correlation markers of anaplastic changes in the esophagus epithelium. The material was taken for cytological examination by maceration with a probe-cytobrush type D1 on Days 1, 7, 10, and 14 of the experiment, and then after every 14 days. The wet smears-imprints of the oral epithelium were fixed in absolute alcohol, hydrated in drinks of a decreasing concentration and distilled water, stained according to Papanicolaou (Gill's hematoxylin, cytoplasmic dyes OG6 and EA50), dehydrated in alcohols of ascending concentration, and cleared in two changes of xylene.

The animals have been withdrawn from the experiment if their bodyweight declined by more than

1/3 or the delay in weight gain by more than 1/3 compared to Control Group I. Outside of these criteria, an equivalent number of animals from the control groups have also been withdrawn from the experiment. The euthanasia of animals was carried out in the following sequence: ether sedation, subcutaneous administration of Zoletil-100 and Xylanit at doses of 8 mg/kg and 4 mg/kg, respectively, provision of vascular access (puncture of the dorsal tail vein), and administration of lidocaine at a dosage of 100 mg/kg [9]. After a 3-fold determination of the circulatory arrest, an autopsy has been performed. The time limit from the moment of clinical death to the beginning of fixation of the histological material was equal to 30 min [10].

The received autopsy material was fixed in 10% neutral buffered formalin, dehydrated in 8 changes, starting with a 50% aqueous solution of isopropanol, and poured into the Histomix paraffin medium with the use of the ESD-2800 embedding module. The thin paraffin sections 4-6 μ m thick, obtained on a semi-automatic rotary microtome, were stained with hematoxylin and eosin. The microscopic examination of the obtained experimental material was carried out with the use of a microscope at low (10x), high (40x) magnifications, and in oil immersion (100x). A digital camera obtained the photomicrographs, the computer processing of the acquired images was carried out in MCview software.

The STATISTICA 13 program (TIBCO Software Inc.) was used for the statistical analysis of obtained results. The mean, median, standard errors, and confidence intervals were calculated using the descriptive program statistics. The Shapiro-Wilk test ($n < 50$) was used to assess the normal distribution of the analyzed quantitative indicators. The aggregates of the variables considered according to the test escaped the law of normal distribution; therefore, nonparametric tests were used for further analysis. The animals' body weight group's mean change and confidence interval were determined using the nonparametric Mann-Whitney U-test. The Kruskal-Wallis H-test was used to compare the frequency of cytopathological changes in the Experimental Groups.

At a significance level of $p < 0.05$, the differences in the compared groups were considered statistically significant. The pairwise comparison was performed using Fisher's exact test if statistically significant differences between the groups.

Findings: The results of measuring the animal's body weight are demonstrated in Figure 1. For the Control Group I and the Experimental Group, the confidence intervals were given at a statistical sig-

nificance level of 95%. The statistically significant ($p < 0.05$) differences in the body weights of animals between the Experimental Groups were obtained on Day 28 of the experiment. The animals from the Experimental Group showed a delay of the body weight gain over the standard variance in the control groups. By Day 42, one animal from the Experimental Group was withdrawn from the study and subjected to euthanasia due to the delay of the

body weight gain by more than 1/3 of the mean value for Control Group I. By Day 70, according to similar criteria, two more animals from the Experimental Group had been withdrawn from the experiment. The pairwise comparison of the mean body weights of animals by Fisher's method revealed statistically significant ($p < 0.05$) differences between the experimental and control groups by the end of the experiment.

Figure 1 – Change of the group mean of the animals' body weight during the experiment

Within the frames of analysis and interpretation of the results of cytological studies, the diagnostic and classification criteria of the Bethesda system were used in part applicable to stratified squamous epithelia [11]. In addition, to draw up a general idea of the cytological picture obtained from these model organisms, the picture of the conditional norm characteristic of Control Group I was reviewed. The typical normal cytological picture for this animal species is shown in Figure 2. The parabasal and intermediate cells of squamous epithelium were found in smears. The cells were round or polygonal in shape, layered on the glass, resulting from which their cytoplasm looked folded. The nuclei had even outlines, approximately the same size, the chromatin was evenly dispersed, and the nucleoli were found in some seats. In the cytoplasm of individual intermediate cells, the glycogen inclusions were found. In Figure 2 and other micrographs of cytological preparations, the nonspecific artifacts of the preanalytical stage were present in the background: mucus, flora, and fragments of plant dietary fibers. They

were ignored until they interfered with an adequate assessment of the drug.

Figure 2 – Smear of the oral cavity epithelium of rats from Control group I on Day 10 of the experiment. Traditional preparation. Papanicolaou's stain. Amplification $\times 980$. Conclusion by Bethesda: No intraepithelial lesions

The nature and frequency of cytopathological changes detected in epithelial smears are summarised in Figure 3. The statistically significant (Kruskall-Wallis H-criteria) differences in the absolute frequency of changes were found between the experimental and control groups ($p=0.005$ for the 1st control, $p=0.031$ for the 2nd control, and $p=0.027$ for the 3rd control). Fisher's method showed a significant difference in the frequency of

squamous epithelium atypia between the experimental and control groups on Day 42 of the experiment. The keratotic and inflammatory changes were presented in both groups. The differences in the frequency of cytopathological changes classified as atypia of squamous epithelium of undetermined significance in the Experimental Group and Control Group II were statistically significant when analyzed using the Fisher's exact test ($p=0.015$).

Notes: ASC-US – the atypical squamous cells of undetermined significance, LSIL – low-grade intraepithelial lesions. The dotted line indicates the total number of animals on the given day of the experiment.

Figure 3 – Absolute frequency of cytopathological changes in Experimental Groups

The atypical squamous epithelium revealed in the study of cytological material is shown in Figures 4 and 5. The atypical cells of undetermined significance had appeared in smears starting from Day 42 of the experiment (Figure 4). According to Bethesda's criteria, the mature squamous epithelial cells with hyperchromic pycnotic nuclei with irregular contours (Figure 4a), chromatin had a rough structure, and dystrophic vacuolization was ob-

served in some cells (Figure 4c). The parabasal cells nuclei were more than twice bigger than the reference ones. They had uneven contours; peaked nuclear membrane created a feeling of the nucleus folding (Figure 4b). The parabasal and mature squamous epithelial cells formed layers. Subsequently, the qualitative changes were noted in smears from the same animals, which we interpreted as the progression of processes.

Figure 4 – Smear of the oral cavity epithelium of a rat from the Experimental Group on Day 42 of the experiment. Traditional preparation. Papanicolaou's stain. Amplification $\times 2450$. Conclusion by Bethesda: Undetermined Atypical Squamous Cells (ASC-US), Atypical Parakeratosis

Figure 5 – Moderate (grade 2) dysplastic changes in the squamous epithelium of the animal's esophagus in the Experimental Group. Thin slicing of the esophagus. Staining with hematoxylin and eosin. Amplification $\times 245$, in selected window $\times 980$

Starting from Day 56 of the experiment, in the smears obtained from the animals of the Experimental Group, the atypical cells were found, described in Bethesda terms as low-grade intraepithelial lesions. The parabasal cells of squamous epithelium with atypical large nuclei (more than three times compared to the reference), anisonucleo-

sis, with variable contours of the nuclear membrane with in one cell, and coarse chromatin were recognized as diagnostically significant. The cytoplasm of parabasal cells with orangeophilous areas reflected the keratinization process. The mature cells have revealed the signs of atypical parakeratosis and dystrophic vacuolization.

The results of histological verification of presumed changes are presented in Figure 5. The disorganization of the squamous epithelium was revealed in the middle third of the esophagus, including derangements of hystoarchitectonics, cell polarity, nuclear polymor-

phism, and sometimes parakeratosis. The stromal reactions included mild lymphohistiocytic infiltration and mucoid swelling. Figure 6 shows the control histological picture: no signs of dysplastic changes in the epithelium, no stromal response, or pathological changes.

Figure 6 – Control autopsy material. Thin slicing of the esophagus. Staining with hematoxylin and eosin.
 Amplification $\times 245$, in selected window $\times 980$

Accordingly, the appearance of the dysplastic changes in the squamous epithelium of esophagus in rats of the Experimental Group occurs on average by Day 70 of the experiment. The cytological changes, corresponding to low-grade intraepithelial lesions, correlate with the histological picture of moderate dysplastic changes of the esophageal epithelium.

Discussion: 4-NQO is a hydrophilic nitroquinoline derivative used in the experimental practice as a mutagen forming DNA adducts (non-covalently bound supermolecular associates) and causing chromosomal aberrations. In particular, 4-NQO is empirically used to model tongue cancer in rats [11]. There is evidence of its toxic effect. Researchers consider the minimum poisonous dose for the application route of administration through the oral mucosa – 20 mg/kg for rats [12, 13].

DMSO – is an amphiphilic aprotic solvent that is miscible with water in any proportion. It is used as

an anti-inflammatory, analgesic agent, and transport system for drug delivery. In biomedical and medico-toxicological studies, the DMSO enhances the permeability of biological barriers for xenobiotics. The toxic doses with systemic effects are very high DMSO concentrations; studies have shown results at doses around 103-105 mg/kg [4].

Experimentally was found that the onset of dysplastic changes in the squamous epithelium of the esophagus occurs on average by Day 70 of the experiment. We consider the LSIL cytopathological conclusion in the Bethesda terms sufficient to interpret the pathological changes as early dysplasia. The morphological criteria for the classification of the identified pathological changes as the intraepithelial lesions and a low-grade dysplasia are changes in the size, shape, contours of nuclei, nuclear-cytoplasmic ratio; disorders of maturation and differentiation of cells, irregular morphology of basal layer, derangements of the cells hystoarchitectonics [14].

In addition, these criteria can act as relevant prognostic markers for staging the pathological process in the course of serial studies.

In this regard, we state that it is necessary to use the markers of cell interactions, adhesion, and migration to verify cytopathological conclusions. To further study the pathomorphological picture of dysplastic changes, it is required to use the methods of immunocytes- and histochemical studies. We assume that in this respect, the promising immunocytochemical markers are cytokeratins and glycoproteins of the CD44 family.

The expression of tight junction proteins, connexins, claudins, E-cadherins was changed in endometriosis and neoplastic diseases of the endometrium [15]. The possibility of using appropriate immunochemical markers for early diagnostics of intraepithelial neoplasia of the oropharynx and esophagus is being investigated.

Conclusion: The correlation and prognostic morphological markers of anaplasia of the esophageal mucosa are represented by dysplastic changes in the epithelium of the oral cavity exposed to the topical effect of a carcinogen. The intensity of morphological changes in the esophagus squamous epithelium towards the precancerous state was significantly higher in the Experimental Group of animals exposed to a long-term effect of the carcinogenic factor, compared to the control groups ($p < 0.05$), which has been confirmed by the results of a comparative histological study.

Thus, a cytological study could be used in clinical practice as a screening tool for precancerous oropharynx and upper esophagus conditions.

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ТҰЖЫРЫМ

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Канцерогенез туындаған жағдайда модельдік организмдерде пайда болатын ас қорыту жүйесінің жоғарғы бөлігінің жалпақ эпителийіндегі өзгерістердің морфологиялық сипаттамасы

Өзектілігі: ісікалды өзгерістерді ерте анықтау хаттамаларын әзірлеу қажеттілігі ас қорыту жолының жоғарғы бөлігінің шырышты қабығының қатерлі ісіктерінің таралуына және уақтылы диагноз қойылмаған кезде осы пациенттердің өмір сүру сапасының төмендеуіне байланысты.

Мақсаты: дәлелденген канцерогендік белсенділігі бар заттарға ұшыраған кезде канцерогенез туындаған жағдайда модельдік организмдерде пайда болатын ас қорыту түтігінің жоғарғы бөлігінің жалпақ эпителийіндегі өзгерістердің морфологиялық сипаттамаларын зерттеу.

Әдістері: Модельдік организмдер ретінде Орехово-Зуев қалалық елді мекенінің ветеринарлық станциясынан алынған сызықты емес ақ егеуқұйрықтардың 40 аналықтары қолданылды. Егеуқұйрықтарды теңдей етіп 4 топқа бөлді. Бірінші бақылау тобы қандай да бір ерекше әсерлерге ұшыраған жоқ. 2–4 топтарда егеуқұйрықтар ауыз қуысының шырышты қабығына седация және механикалық скарификация микротравматизациясын жүргізді, содан кейін аптасына 2 рет жиілікпен 0,9% NaCl ерітіндісін (2-бақылау тобы), 1% диметилсульфоксидтің сулы ерітіндісін (DMSO, Акрихин, Ресей) (3-бақылау тобы) немесе 0,1 мг/мл 4-нитрохинолин-N-оксиді (4-NQO, Acros Organics, АҚШ) (4-тәжірибе тобы) немесе 1% DMSO сулы ерітіндісін қолданады.

Нәтижелері: ас қорыту жүйесінің жоғарғы бөлігінің шырышты қабығының созылмалы жарақатымен байланысты Цитологиялық көрініс спецификалық емес реакциялармен сипатталды: қабыну, гипер- және паракератоз, атрофиялық өзгерістер. Негізгі және бақылау топтары арасында мұндай өзгерістердің пайда болу жиілігіндегі статистикалық сенімді айырмашылықтар анықталған жоқ. Канцерогендік белсенділігі бар субстанцияның созылмалы әсері бар негізгі топтағы ісік алды жай-күйі бағытындағы жалпақ эпителийдің ерекше өзгерістері бақылау топтарымен ($p < 0,05$) салыстырғанда дұрыс тастағанмен кездесті және салыстырмалы гистологиялық верификация нәтижелерімен расталды.

Қорытынды: Цитологиялық зерттеуді клиникада орофаринс пен Жоғарғы өңештің қатерлі ісікке дейінгі жағдайларын скрининг ретінде зерттеу үшін ұсынуға болады.

Түйінді сөздер: қатерлі ісіктер, ас қорыту жүйесі, цитология, эпителий, сквамозды жасушалық дисплазия, эксперименттік модель.

АННОТАЦИЯ

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Морфологическая характеристика изменений плоского эпителия верхнего отдела пищеварительного тракта, возникающих в модельных организмах в условиях провоцированного канцерогенеза

Актуальность: Необходимость разработки протоколов раннего выявления предопухолевых изменений обусловлена распространенностью злокачественных новообразований слизистой оболочки пищевода и снижением качества жизни этих пациентов при несвоевременной диагностике.

Цель: Исследование морфологической характеристики изменений плоского эпителия пищевода, возникающих в модельных организмах в условиях провоцированного канцерогенеза при воздействии субстанций с доказанной канцерогенной активностью.

Методы: В качестве модельных организмов использовали 40 самок нелинейных белых крыс, полученных из ветеринарной станции Орехово-Зуевского городского поселения. Крысы были распределены на 4 равные группы. Первая контрольная группа не подвергалась какому-либо специфическому воздействию. В группах 2–4 крысам проводили седацию и механическую скарификационную микротравматизацию слизистой полости рта, а затем аппликационно с частотой 2 раза в неделю наносили 0,9% раствор NaCl (контрольная группа 2), 1% водный раствор диметилсульфоксида (DMSO, Акрихин, Россия) (контрольная группа 3), или 1% водный раствор DMSO, содержащий 0,1 мг/мл 4-нитрохинолин-N-оксид (4-NQO, Acros Organics, США) (опытная группа 4).

Результаты: Корреляционно-прогностические морфологические маркеры анаплазии слизистой оболочки пищевода представлены диспластическими изменениями эпителия полости рта, подвергшегося топическому воздействию канцерогена. Выраженность морфологических изменений в плоском эпителии пищевода в направлении предракового состояния достоверно выше в опытной группе при длительном воздействии канцерогенного фактора по сравнению с контрольными группами ($p < 0,05$), что подтверждалось результатами сравнительного гистологического исследования.

Заключение: Цитологическое исследование может быть предложено для изучения в клинике в качестве скрининга предраковых состояний верхнего отдела пищевода.

Ключевые слова: злокачественные новообразования, пищеварительная система, цитология, эпителий, плоскоклеточная дисплазия, экспериментальная модель.

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