Revista da Faculdade de Odontologia de Porto Alegre
V. 63, n. 2 (2022) - Artigos Originais
DOI: 10.22456/2177-0018.125090

Transmission electron microscopy in oral cytopathology: a pilot study

Microscopia eletrônica de transmissão em citopatologia bucal: um estudo piloto

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ABSTRACT

Introduction: Cytopathology is a collection method that allows cell analysis through the different techniques. The oral mucosa exfoliated cells observation demonstrates morphological, biochemical and/or molecular aspects depending on the type of processing of the sample. **Aim:** This study tested the use of oral cytopathology associated with transmission electron microscopy (TEM) to observe the morphology of cells, mainly in relation to the cell nucleus, the cytoplasmic membrane, and cell junctions. **Materials and Methods**: Exfoliated epithelial cells from the oral mucosa were analyzed by TEM from individuals exposed to tobacco and alcohol, with leukoplakia or with a histopathological diagnosis of squamous cell carcinoma. **Results**: The cytoplasmic cell-cell junctions in the malignant samples lost the characteristic irregular pattern formed by the numerous interdigitations and the junctional process of normal cells and started to present a straight cytoplasmic surface. The nuclei of cells from leukoplakia and squamous cell carcinoma samples showed heterogeneous staining, while non-lesional cells were homogeneous. **Discussion:** The analysis of oral cytopathological smears by TEM contributes to the understanding of the changes that occur during the process of malignancy of the oral mucosa, especially with regard to the cytoplasmic membrane and intercellular junctions. **Conclusion**: TEM may be a good analytical method to investigate morphological changes in exfoliated cells of the oral epithelium.

Keywords: Cytological techniques. Mouth mucosa. Transmission electron microscopy. Squamous cell carcinoma.

RESUMO

Introdução: A citopatologia é um método de coleta que permite a análise celular por meio de diferentes técnicas. A observação das células esfoliadas da mucosa bucal demonstra aspectos morfológicos, bioquímicos e/ou moleculares dependendo do tipo do processamento empregado. **Objetivo**: Este estudo testou o emprego da técnica de citopatologia bucal associada à microscopia eletrônica de transmissão (MET) para observar a morfologia das células, principalmente com relação à membrana citoplasmática, as junções celulares e ao núcleo da célula. **Materiais e Métodos:** Células epiteliais esfoliadas da mucosa bucal foram analisadas por MET de indivíduos expostos a tabaco e álcool, apresentando leucoplasia ou com diagnóstico de carcinoma espinocelular. **Resultados:** As junções citoplasmáticas célula-célula nas amostras malignas perderam o padrão irregular característico formado pelas inúmeras interdigitações e o processo juncional das células normais e passaram a apresentar uma superfície citoplasmática reta. O núcleo das células das amostras de leucoplasia e do carcinoma espinocelular apresentou coloração heterogênea, enquanto as células não lesionais foram homogêneas. **Discussão:** A análise de esfregaços citopatológicos bucais por MET contribui para o entendimento das alterações que ocorrem durante o processo de malignidade da mucosa bucal, principalmente no que diz respeito à membrana citoplasmática e as junções intercelulares. **Conclusão:** A MET pode ser um bom método analítico para investigar alterações morfológicas em células esfoliadas do epitélio bucal.

Palavras-chave: Técnicas citológicas. Mucosa bucal. Microscopia eletrônica de transmissão. Carcinoma de células escamosas.

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Data de envio: 07/06/2022 | **Data de aceite:** 14/07/2022

Introduction

Ultrastructural examination by Transmission Electron Microscopy (TEM) was performed in non-oral cytopathological samples such as lymph node, bronchi alveolar lavage, pleural fluid, and urine smears collected via fine needle aspiration and exfoliative cytology[1].

Matravers and Tyldesley evaluating scraped normal human oral and squamous carcinomas cells by Scanning Electron Microscopy (SEM) detected alterations in cell surface morphology[2]. Chomette *et al.*[3] using the same collection method and technique could observe significant changes between healthy, dysplastic and tumoral cells and recommended this methodology for distinguishing between them.

Concerning histological samples, oral leukoplakia (OL) evaluated by TEM showed discontinuous basal lamina, ruptured hemidesmosomes, and pathologic cytoplasmic processes[4]. The changes in oral squamous cell carcinoma (OSCC) were: irregular borders of nuclei, prominent nucleolus, abundant cytoplasmic projections, and decreased or total loss of hemidesmosomes[5].

It is possible to state that oral samples associated with electron microscopy are able to observed changes in the ultrastructure between normal and lesions oral epithelial cells[2-5]. However, these findings were studied only in cytosmears by SEM or in tissue specimens by TEM. This study aims evaluate if TEM can be a good tool to observe the ultrastructural morphology of exfoliated oral epithelial cells.

Materials and methods

The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee at the UFRGS under protocol number 29423. The subjects were healthy male patients, older than 18 years. All individuals signed an informed consent form. The Control Group (CG) comprised individuals not exposed to risk factors and with nonclinical lesions; the Alcohol-Tobacco Group (ATG) comprised alcohol and tobacco users without clinical lesions; OL, and OSCC lesions were analyzed. The inclusion and exclusion criteria were the same ones as previously established[6].

Cytopathological smears were collected with rotating movements using a cytobrush (Absorve®, São Paulo, Brasil) in the border of the tongue in non-lesional groups, over OL, and adjacent to OSCC. The cytobrush was stored in an Eppendorf® with 1.5 mL of glutaraldehyde solution [glutaraldehyde 0.5% (Sigma Chemical Co., Missouri), paraformaldehyde 4% (Reagen, Brazil), and a 0.2 M sodium phosphate buffer (SPB), final pH solution 7.4] at 4 °C until the samples were processed. The collected sample was centrifuged to form a pellet. The samples were washed with SPB (0.1 M, three times for 15 minutes each) and postfixed in 1% OsO4 (Sigma Co.) in 0.1 M SPB for 1 hour. The samples were washed with 0.1 M SPB, dehydrated in a graded series of acetone, embedded in resin (Durcupan®; ACM-Fluka, Switzerland), and polymerized at 60 °C. In each of the steps above, the cells were pelleted by centrifuging the vials at 3000 rpm for 5 minutes and the excess reagent was removed. Ultrathin cross-sections (80–100 nm) were obtained using an ultramicrotome (MT 6000-XL; RMC, Tucson, Arizona) and a freshly-made glass knife. The ultrathin sections were placed on copper grids and stained with 2% uranyl acetate for 20 minutes and lead citrate in distilled water for 10 minutes. The analysis was performed by TEM (JEM 1200 EX II) at 80 kV voltages.

For each sample, five cells were captured in a magnification which allowed complete visualization of the cell (± 5K). The magnification was then increased to verify the details in the cellular membrane (± 50K).

Results

The sample comprised four individuals of CG, three of ATG, one of OL and one of OSCC. The median age was 50,6 years (32,0 – 65,0). In regarding to alcohol, the ATG ingests beer, vodka, whisky and cachaça; the OL individual ingests 4 liters of beer or wine/week; and the OSCC individual ingests 3 liters "cachaça"/day. Concerning tobacco, the ATG smokes a median of 26,6 (20 – 40) cigarettes/day for 32,6 (18 – 40) years; the OL individual has smoked 60 cigarettes/day for 50 years and the OSCC individual has smoked 20 hand-rolled cigarettes/day for 43 years. Table 1 summarizes characteristics of the sample. The common characteristic for all samples was the absence of cytoplasmic organelles; a detailed cytomorphological report is shown in table 2.

Table 1: Characteristics of the sample.

	Group	Age	Site	Tobacco	Alcohol
1	CG	32	border of the tongue	NO	≤ once/week
2	CG	43	border of the tongue	NO	≤ once/week
3	CG	50	border of the tongue	NO	≤ once/week
4	CG	63	border of the tongue	NO	≤ once/week
5	ATG	33	border of the tongue	20 cigarettes/day for 18 years	3 L beer/day
6	ATG	52	border of the tongue	40 cigarettes/day for 40 years	400 mL vodka or whisky/week
7	ATG	65	border of the tongue	20 cigarettes/day for 40 years	300 mL "cachaça"/week
8	OLG	64	Floor of mouth	60 cigarettes/day for 50 years	4L beer or wine /week
9	SCCG	54	Ventral tongue/Floor of mouth	20 "palheiros"/day for 43 years	500 mL "cachaça"/day

Table 2: Cytomorphological differences between Control, Alcohol-Tobacco Group, Oral Leukoplakia, and Oral Squamous Cell Carcinoma oral exfoliated cells.

	Control group (Figure 1A, 1B, 1C, 1D)	Alcohol-tobacco group (Figure 1D, 1E, 1F, 1G)	Oral leukoplakia (Figure 2C, 2D, 2E)	Oral squamous cell carcinoma (Figure 2H, 2I, 2J)
Celular morphology	Normal	Without alterations	Elongated and thin; most of the cells were anucleated	Increased nuclear/cytoplasmic ratio Pleomorphic cells
Cytoplasmic membrane	Well-defined microvilli and multiple cellular junctions with desmosomes	Irregular with desmosomes	Well-defined microvilli and a few desmosomes	Without microvilli and absence of cell junctions
Nucleus	Dark colored and homogeneous	Slightly heterogeneous	When present, heterogeneous	Heterogeneous
Junctional complex number	Normal	Slightly decreased	Decreased	Absent
Intercellular space	Without alterations	Slightly increased	Incresead	Strongly increased
Other findings	Bacterial colonies, lipid particles, tonofilaments, and keratohyalin granules	Tonofilaments and keratohyalin granules	Keratohyalin granules	Intracellular vacuolization (Laking Effect)

The normal cells presented cellular adhesion by desmosomal junctional process, cytoplasmatic membrane showing microvillus, and the nucleus presented dark homogeneous color with regular nuclear membrane. The alcohol-tobacco group cells showed cells attached with minimum intercellular space, cytoplasmatic membrane with keratohyalin granules, irregular nuclear membrane, heterogeneous nuclear content, and decreased of junctional complex (Figure 1).

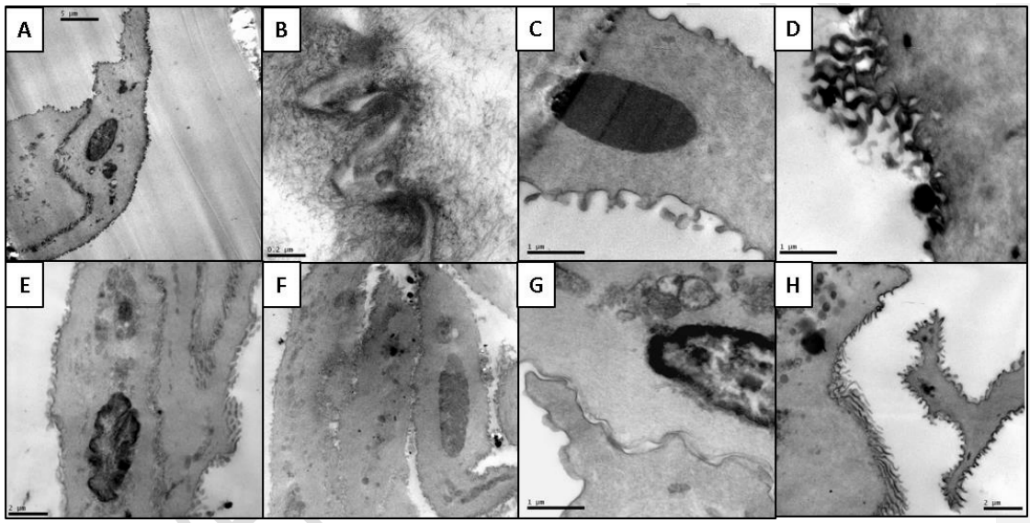

Figure 1: Control group oral epithelial cells sample: A) cellular adhesion B) desmosomal process C) homogeneous nucleus D) microvillus. Alcohol-Tobacco Group oral epithelial cells sample: E-F) Multiple cells attached G) Heterogeneous nuclear content, cytoplasmatic membranes without junctional complex, H) Microvillus. TEM, uranyl acetate, and lead citrate stains.

Figure 2 shows the clinical aspect, histological photomicrography and cytosmears analysed by TEM of oral leukoplakia and OSCC. These cells showed heterogeneous nuclear content and irregular cytoplasmatic membrane with absence of junctional complex. Figure 3 summarize all results in illustrated diagram.

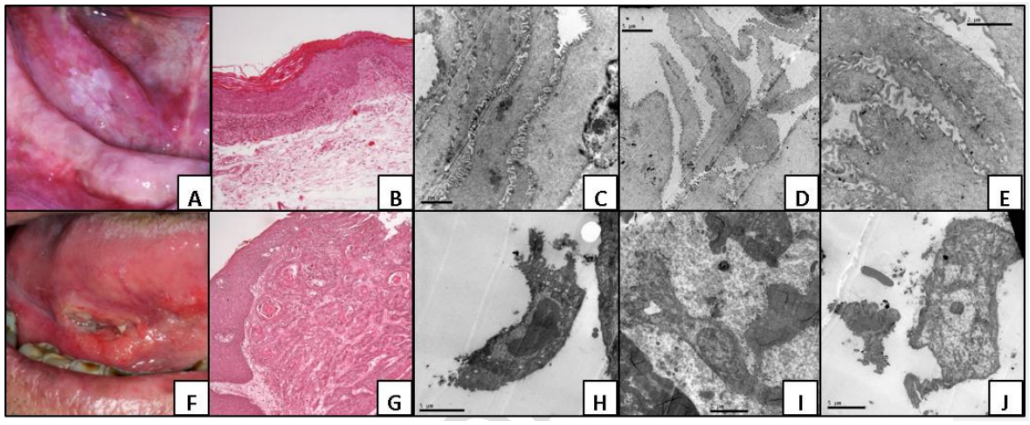

Figure 2: Leukoplakia samples: A) Clinical image B) Squamous oral hyperortokeratinizated epithelia (HE, 10x), C-E) Multiple elongated cells attached with intercellular space increased and keratohyalin granules. Squamous cells carcinoma samples: F) Clinical image G) Malignant proliferation of epithelial squamous cell (HE, 10x). H-I) Pleomorphic epithelial cells J) Laking effect. TEM, uranyl acetate, and lead citrate stains.

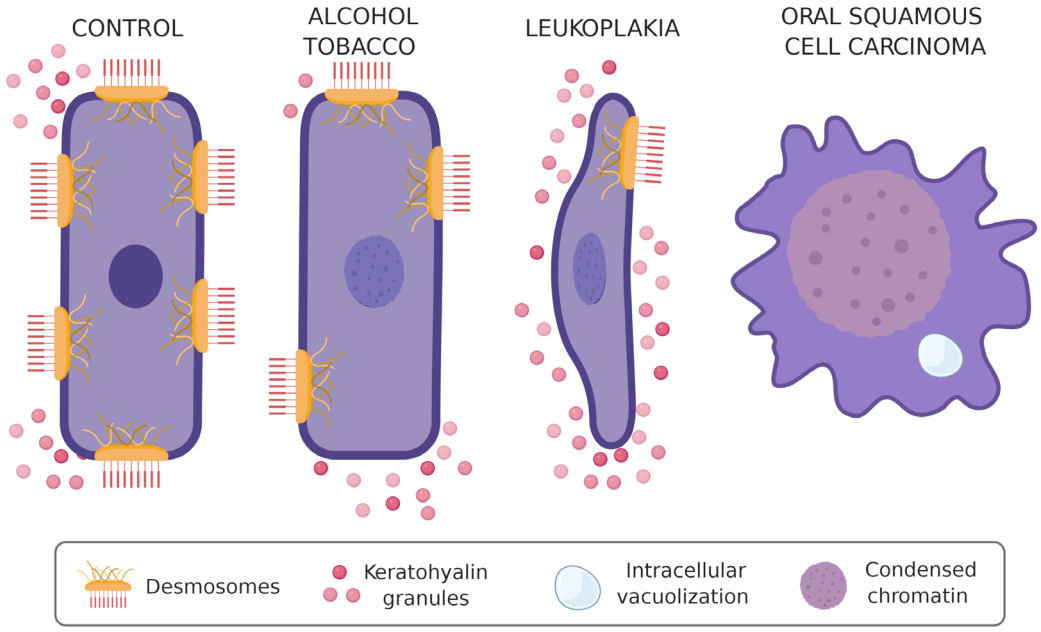

Figure 3: Diagram with the main differences between the analyzed samples.

Discussion

The oral cytopathology associated with TEM analysis was used successfully in this study to demonstrate morphological differences among normal, exposed carcinogens, potentially malignant and malignant cells. There is strong evidence that this is the first research that shows ultrastructural changes in oral exfoliated cells analyzed by TEM. Other studies have already reported changes in the oral mucosa by TEM, however the samples used were tissues. Moreover, some studies are outdated[2-5,7].

In this study, a gradual loss of cellular junctions and an increase in intercellular spaces were observed in ATG and OL followed by OSCC, respectively. This was found in histological analysis/TEM by Frithiof with a decrease in the number and size of desmosomes in OSCC cells[7]; by Cheng and Hudson[5] in normal epithelia adjacent tumor and OSCC; by Tamgadge *et al.*[4], and recently by Olinici *et al.*[8] in leukoplakias. The loss of cell adhesion results in increased tissue permeability, which exposes cells from all layers to carcinogenic agents[9].

Nuclear atypia, increase of the nuclear volume with multiple nucleoli, and nucleolar margination were observed in OL in previous studies[4,8]. Our results show a gradual loss of homogeneity nuclear and nuclear membrane irregularity with increased condensed chromatin in CG, ATG, OL and OSCC, respectively. Irregular nuclear materials can indicate early apoptotic events, and evident nucleolus could mean disturbance in cellular proliferation[10,11].

The Lacking effect is considered a degenerative change observed previously in leukoplakia tissues[4]. In this study, this phenomenon was observed in OSCC, reinforcing the signal of cellular alterations in disease conditions.

Keratohyalin granules (KG) are decreased or absent in non-homogenous leukoplakia, contrasting with homogenous that presented increased number and size of KG[4]. Comparing with our cases, CG, ATG and OL (homogeneous) presented KG while OSCC (ulcerated) did not present this component, probably because the cornea and granular layer have been lost.

The analyzed sample does not allow to extrapolate the results to a population, so larger samples are necessary to confirm these preliminary findings. In addition, it is known

that this methodology would not be applicable to clinical routine, not only because of the technical complexity in preparing MET smears, but also because of its cost. In conclusion, these results reinforce the theoretical descriptions about chemical/morphological changes that occur in leukoplakia and OSCC.

Conclusion

TEM may be a good tool to evaluate oral exfoliated epithelial cells, complementing light microscopy. The ultrastructural details confirm the changes, especially decreased epithelial junction in potentially malignant disorders and in oral cancer comparing with normal cells.

Acknowledgments

The authors thank Sílvia Barbosa and Maria Cristina Faccioni Heuser for consulting in the laboratory technical aspects of the experiments.

Funding

This work was supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Ministery of Education, Brazil).

Conflict of interests

The authors declare that they have no conflict of interest.

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