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Optimization of Alkaline Single Cell Gel Electropohoresis (Comet Assay) Protocol for Cells from Oral Cavity

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Authors' contributions

This work was carried out in collaboration between all authors. Author EAPG designed the study, cooperated with the technical work and wrote the final version of the document. Author VVM developed the protocol, managed the literature searches, wrote the first draft of the manuscript and cooperated with the final version. Authors EAPG and VVM managed the analyses of the study. Author AMP performed the statistical analysis and cooperated in the correction of the final version. All authors read and approved the final manuscript.

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ABSTRACT

Comet assays or single cell gel electrophoresis for detection of DNA damage is a test that has been widely utilized to assess the effects of expositions to environmental genotoxicants. The test is also used to evaluate DNA damage related to chronic inflammation or preneoplastic and neoplastic conditions. The cells more frequently assessed in comet assay in humans are the peripheral blood lymphocytes, but there are other cell types that have been considered for that purpose. Among those, buccal cells have received attention for its suitability for comet assay, but there have been

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relatively few studies on comet assay in buccal epithelial cells. However, there are technical difficulties related to comet assay in buccal epithelial cells that justifies the attempts to develop or optimize protocols that could contribute to standardize the test allowing more widespread use of buccal cells in biomonitoring or clinical trials. In the present work, we compared three protocols: the standard technique of alkaline comet from Tice et al. 1999 and the protocols developed specifically for oral cavity cells from Valverde et al. 1997 and Szeto et al. 2005. We introduced modifications in the protocols related to, a device utilized to scrape the cells from the mucosa, the place and volume of sample enzymatic digestion, trypsin concentration, and also, the times for lysis incubation and unwinding. This modified protocol is a contribution to the optimization of comet assay for buccal cells and contributes to its utilization in biomonitoring human DNA damage.

Keywords: Comet assay; buccal cells; genotoxicity; single cell electrophoresis.

1. INTRODUCTION

The alkaline Comet Assay is a technical procedure utilized in order to detect different lesions in the DNA molecule, like single strand breaks, alkali labile sites and crosslinks. Cells that are usually evaluated in humans are peripheral blood lymphocytes [1,2]. Sampling those cells in humans requires venipuncture or digital puncture, procedures that can be considered invasive [3].

In recent years a number of studies have been developed with comet assay from several cells sources; that included exfoliated cells, from bladder, gut, nasal epithelium, and breast and cervix uteri among others [4-7].

Oral cells are capable of metabolizing a large amount of carcinogens [8], these feature also define these cells as adequate biomonitoring sensors for genotoxicity evaluations [4,9,7,10,11].

This study was done in order to compare several technical procedures in order to achieve a better disaggregation of epithelial cells and to improve their lysis so as to increase cell yield and proportion of uniformly stained and contoured nucleoids (USCN) useful for DNA damage evaluation. We propose that the results presented here would contribute to optimize this widely used procedure for genetic biomonitoring.

2. MATERIALS AND METHODS

2.1 Subjects

There were nine healthy donors (7 females and 2 males). Protocols number 1, 2 and 3 were applied to cells obtained from 8 subjects (6 females and 2 males, age 23 to 35 years); in the evaluation of Protocol No. 4 there were 9 subjects (7 females and 2 males age 21-33 years). All were selected under the following inclusion criteria: no previous viral infection during the month previous to sampling, non-smoking habits, not suffering periodontal disease or wearing removable dentures, nor chronically use of therapeutically drugs, neither exposition to known genotoxicants. Oral health was previously evaluated in all participants by a trained professional (V.V.M), in order to exclude the presence of lichen, leukoplaquia or inflammatory lesions.

2.2 Sample Collection

Buccal cells were collected with a wooden stick in the protocol from Valverde et al. [12], a soft toothbrush for that of Szeto et al. [13]. In our protocol and in that of Tice and Vasquez, [14], a wooden Ayre spatula was used. The spatula was utilized in the inverted position in order to scrape with the grip Sampling was done by a gentle scraping over each cheek mucosa and repeated twenty times, being each of these samples extended in one slide.

Prior to cell collection the subjects were instructed to rinse their mouths with saline solution (0, 9%) for 2 minutes. Participants were asked not to eat, chew gum or drink any beverage except non-carbonated water in the hour previous to sampling. Lipstick, if used, should be removed two hours before. Then the spatulas with the samples were placed in 15 ml Falcon flasks containing 4 ml of PBS at 4°C and each were vortexed during 20-25 seconds, after which the samples were centrifuged at 1000 rpm for 10 minutes at 4°C.

2.3 Positive Control

An aliquot of 10^5 cells was treated with 50 µM hydrogen peroxide at 4°C for 5 minutes and used

as positive control for each protocol to verify the responsiveness to the oxidant treatment.

2.4 Evaluation Criteria of Technical Protocols

Four technical protocols were compared: Valverde et al. (protocol 1) [12]; Tice and Vasquez, (protocol 2) [14]; Szeto et al. (protocol 3) [13] and ours (protocol 4). Variables considered for comparison were: i) number of epithelial origin`s identifiable cells; ii) number of living cells; iii) number of nucleoids by 400x microscopy field; iv) regularity of comet head contour expressed as USCN, so as to evaluate the homogeneity of cell lysis; v) basal DNA damage and DNA damage after hydrogen peroxide treatment. (See Table 1)

2.5 Staining and Scoring

Dry slides were stained with ethidium bromide (0.02 mg/mL) before examination under a fluorescence microscope (Carl Zeiss), (excitation filter: 515-560 nm; barrier filter = 590 nm), at magnifications of 400x or 1000x. Fifty cells per slide were analyzed. Scoring was performed by the same person and it was a blind analysis regarding the origin of the protocol that produced each slide. Classification of comets includes five arbitrary damage levels according to the amount of DNA in the "comet" tail: level 0: no damage; 1: low damage, 5-20%; level 2: medium damage, 20-40%; level 3: high damage, 40-90%. Cells in level 3 and 4 were considered damaged. "Comets" with more than 50% of the material in the tail and no nuclei detectable was classified as "clouds" and were not scored [15].

2.6 Index of DNA Damage Calculation

The Index of damage is calculated by multiplying the value of the visual scoring of the category (damage from 0 to 4) by the number of nucleoids (comets) classified in each category:

$$ID = n0\,(0) + n1\,(1) + n2\,(2) + n3\,(3) + n4\,(4)$$

Where n = number of cells in the damage level [15]

2.7 Application of the Modified Protocol (4) to Vero Cell Line

Our modified protocol (4) and the original protocol of Tice and Vasquez, (protocol1) [14] were also applied to Vero cells in order to verify whether the introduction of additional steps than those in protocol 1 could increase the *ID*. Vero cells grow adherent and therefore cultures must be treated with trypsin to detach the cells that were kept in culture for 72 hours in an incubator with 5% CO_2 at 37℃. Cells were seeded in 5 cm Petri dishes at 1.0×10^5 cells/mL in Dulbecco MEM (DMEM) containing 10% calf serum, penicillin (100 unit/mL) and streptomycin (100 µg/mL). Buccal epithelial and Vero cells were treated at 4°C for 5 minutes with hydrogen peroxide at increasing concentrations (0, 50, 100 µM) to evaluate if DNA migration in response to oxidative challenge could be influenced by the protocol applied.

2.8 Statistical Analysis

In order to determine the *Index of DNA damage (ID)* for each treatment 10 slides, 50 comets scored in each slide, (500 comets) were selected from the assays 3 and 4 in each protocol. X^2 test was utilized and the Pearson correlation coefficient was applied when Damage Index in buccal and Vero cells, processed according to protocol 4 was compared [16]. Data obtained for nucleoids per field (x400), baseline DNA damage index, H_2O_2 treated DNA damage index were analyzed by comparing the mean ± 0.95 confidence interval in each of the four protocols. The overlap of the intervals indicates non significant differences between the means.

3. RESULTS

Table 2 and Fig. 1 summarize the results obtained with the four different protocols. When the Tice and Vasquez, [14] protocol was applied (protocol 1), from 3 to 5 nucleoids were obtained per 400X field, but 86.1% of all nucleoids were rounded and complete with no tail at all and the amount of DNA migration resulted in 31.1 ± 5.1 DI, since most of the nucleoids seemed to be in damage level 0 or 2. Buccal cells aliquot that were incubated with 50 µM hydrogen peroxide, did not showed migration in two third of the cells and the damage index (30,3 ± 4), did not increase but was slightly lower than the basal damage.

In protocol 2 [12], proteinase K digestion was performed for 60 or 30 minutes at 37°C with the cells embedded in agarose, and then slides were subsequently incubated 20 minutes for unwinding at pH 13.0. Microscope observations

at 400X and 1000X allowed to see only a few empty nucleoids with scarce DNA amount and almost null DNA tails, (< 1 in 10 successive 400X fields) also many clouds were seen (results did not allow ID calculation). When cells were treated with 50 µM hydrogen peroxide (5 min 4°C), the amount of intact nucleoids did not vary (results did not allow *ID* calculation).

In the assays that were performed after protocol 3 [13], procedure included double consecutive digestion that was done in two different ways, the first assay: 300 µl of (0.025%) trypsin in a 1.5 ml tube during 30 minutes at 37°C. In the second and third assays, 50 µl of trypsin at the same concentration was poured over the slides with agarose embedded cells, also for 30 minutes at 37°C; followed by digestion with (1 mg/ml) proteinase K over the slide during 60 minutes at 56°C. Cells that were digested in suspension, were subsequently embedded in LMPA and then incubated for unwind. While those cells whose digestion was performed on the slides, incubation for unwinding, proceeds immediately after. Both unwinding treatment were at pH 9.1.

Results from three assays in protocol 3 were as follows: in the first assay a high proportion (83%) of incomplete not evenly rounded nucleoids were seen and also abundant clouds were obtained. See Fig. 2. When DNA migrates into comet tail, the resulting images of different degrees of damage, are as can be seen in Fig. 3. While in the second and third assays, the overall proportion of USCN rose from 17% to 27%, (a mean of 0. 8 USCN per each 400x field was observed. In this protocol the baseline DNA damage index, that was calculated from results obtained in assays third and fourth, yielded a DI = 92 ± 18,4. While In the cells exposed to 50 uM hydrogen peroxide, damage index increased to 103 ± 25, 7. The increase was not significant ($p>0.05$). See Fig. 4.

In the protocol 4, the overall cells yield according to the scrapping procedure was: Ayre spatula: 1,5 to 3,7 $x10^5$ cells, mean 2.1 x 10^5 cells, *versus* toothbrush: 0.5 to 2,3 x 10^5 cells, mean 0.94 x 10^5 cells. Cheeks scraps with the inverted Ayre spatula resulted in a higher number of viable cells (23%) when compared to that detached with tootbrushes (14%).

The 400X observation allowed seeing an average of 6 USCN per each 400X field. The proportion of USCN reached a 41% what doubles the number of evaluable nucleoids obtained with protocol 3. DNA migration in untreated buccal cells resulted in a basal damage index of 61.7 ± 5.56. In the aliquot exposed to 50 uM hydrogen peroxide the damage index rises to 84,1 ± 6,56. The increase was significant ($p<0.05$) and was in the range of response to H_2O_2 treatment reported by others [17].

An overall comparison of the evaluated variables is summarized in Table 2. The greater amount of USCN in protocol 1 is counteracted with the lack of response to hydrogen peroxide treatment. Protocols 3 and 4 exhibits the best results, but with protocol 4 there is a greater yield of analyzable cells (USCN) a lower DNA damage base line and a greater responsiveness to the oxidant treatment.

Buccal mucosa cells and Vero cells not treated with peroxide did not differ significantly at baseline DI, while increased their DI in a dose dependent-manner after challenging with hydrogen peroxide. In oral mucosa cells, the aliquot treated with hydrogen peroxide increased its *DI* more than did the Vero cells when both were evaluated with the 4th protocol but the difference was not significant ($p> 0.05$). See Fig. 5.

4. DISCUSSION

The variety of cell types in which this technique can be applied; including intestinal, bronchial and bladder among others [18], is relevant to obtain a more direct correlation between the type and amount of DNA damage with their biological significance for certain clinical conditions [4,5,7,19]. This could lead to an increase in the use and utility of this technique in cancer prevention. Tumors originated in the mucosal areas of the mouth, salivary glands, oro, naso and hypopharynx, exhibit a high rate of morbidity and mortality with a high annual incidence on a global scale [20].

These issues have contributed to the interest in the use of oral epithelial as biosensors for genotoxicants. However, there are technical difficulties associated with the isolation of these cells from the buccal cavity. Cells in the lining epithelium are tightly bound together so that the structures responsible for such intercellular junctions must be mechanically broken and enzymatically digested to obtain isolated living cells for Comet assay.

Table 1. Comparison between protocols for comet assay in cells from oral cavity

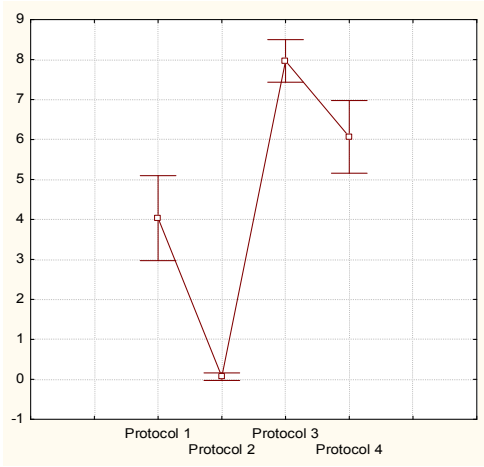
Procedure steps	Protocol 1. Tice and Vasquez 1999	Protocol 2. Valverde et al 1997	Protocol 3. Szeto et al 2005	Protocol 4. Modified by authors
Sampling	Scrapings both cheeks with Ayre spatula. Vortexing in 15 ml Falcon flask with 4 ml of PBS.	Scraping both cheeks with a wooden stick. Vortex in 15 ml Falcon flask with 4 ml of PBS.	Sampling of both cheeks with soft toothbrush. Vortex in 15 ml Falcon flask with 4 ml of PBS.	Sampling of both cheeks (20 times each) with an Ayre spatula. Vortex 25 seconds in 15 ml Falcon flask with 4 ml of PBS.
Centrifugation	1000 rpm , 10 min at 4°C	2500 rpm for 5 min at 4°C	2500 rpm, 10 min at 4°C.	1000 rpm, 10 min. 4oC
Agarose cell suspension	10 µl of cells in PBS plus 75 µl LMPA in 1,5 ml tube.			
Cell lysis	2.5M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton-X (pH10) at 4°C. Duration: 60 minutes.	2.5M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton-X (pH 10) at 4°C. Duration: 24 hours	2,5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton-X (pH 10). At 4°C. Duration: 60 minutes	2,5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton-X (pH 10). At 4°C. Duration: 7 days.
Tripsin digestion	Not done	Not done	300 µl in Trypsin buffer(50 mM Tris, 1mM CaCl2 pH 7.8) in 1.5 ml Eppendorf tube 0.25% at 37ºC30 minutes(second variant in the protocol) Digestion with Typsin poured over the slides	0.05% in 200 µl Trypsin buffer(50 mM Tris, 1mM $CaCl_2$ pH 7.8) in 1.5 ml Eppendorf tube at 37ºC. 30 minutes
Proteinase K digestion	Not done	100 µl PK (10 mg/ml) in PK buffer (10% SDS, 50 mM EDTA, 10 mM Tris-Cl (pH 7.4) at 37°C. 30 minutes (second variant in the protocol); digestion during 60 minutes	100 µl PK (1 mg/ml) in PK buffer (10% SDS, 50 mM EDTA, 10 mM Tris-Cl (pH 7.4) in 1.5 ml Eppendorf tube at 37°C. 60 minutes (second variant in the protocol) Digestion with PK poured over the slide	500 µl PK (1 mg/ml) in PK buffer (10% SDS, 50 mM EDTA, 10 mM Tris-Cl (pH 7.4) in 1.5 ml Eppendorf tube at 37°C. 60 minutes
Alkaline treatment	(300 mM NaOH / 1 mM EDTA), pH 13. at 20°C. 20 minutes	30 mM NaOH /1 mM Na_2EDTA, pH 13. At 20°C. 20 minutes	10 mM NaOH, 1mM EDTA, pH 9.1. At 20°C. 20 minutes	10 mM NaOH, 1mM EDTA, pH 9.1,20 min. At 20oC. Duration: 25 minutes
Electrophoresis conditions	(300 mM NaOH / 1 mM EDTA)	30 mM NaOH /1 mM Na2EDTA, pH 13.0.8V.cm^{-1}.	10 mM NaOH, 1mM EDTA, pH 9.1 0.9 V.cm^{-1}. 20°C. 18 minutes	10 mM NaOH, 1mM EDTA, pH 9.1

Procedure steps	Protocol 1. Tice and Vasquez 1999	Protocol 2. Valverde et al 1997	Protocol 3. Szeto et al 2005	Protocol 4. Modified by authors
	0.8 V.cm^{-1}. 20 minutes at 20°C.	20 minutes at 20°C		0.9 V.cm-1. 20°C. 20 minutes
Neutralization	dH_2O + Neutralization solution: 0.4 Tris-HCl pH 7.5.	dH_2O + Neutralization solution: 0.4 Tris-HCl pH 7.5.	dH2O + Neutralization solution: 0.4 Tris-HCL pH 7.5	dH_2O + Neutralization solution: 0.4 Tris-HCL pH 7.5
Staining	Ethidium bromide (0.02 mg/mL)	Ethidium bromide (0.02 mg/mL)	Ethidium bromide (0.02 mg/mL)	Ehidium bromide (0.02 mg/mL)
Results	Abundant nucleoids without lysis in more than 80%. Low migration. Most of nucleoids between 0 and II according to the five level scale.	Incomplete nucleoids. DNA with irregular migration. (Results from the second variant in the protocol) Lysed cells. Free DNA migrated Scarce nucleoids (< 1 x 10 400x fields)	Disintegrated nucleoids alternating with intact ones. Nucleoids with DNA leakage as strings. Detachment agarose gel slides. USCN. (17%) (second variant in the protocol) Nucleoids with DNA leakage as strings. USCN. (27%)	Lysed cells, presence of uniformly contoured nucleoids. Head and tail easy identifiable. A mean of 6 USCN for each 400x field were observed

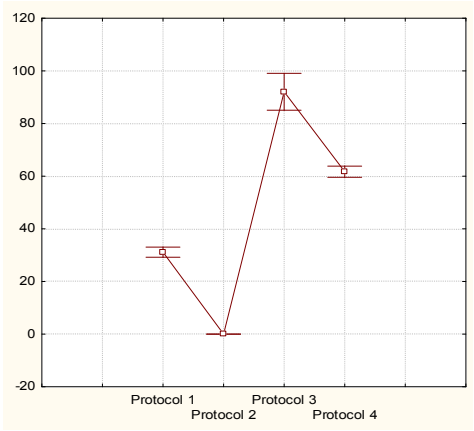
Table 2. Comparison of data obtained with the different protocols

	Protocol	Mean	SD[d]	SE[e]	Min.[f]	Max.[g]
Nucleoids per field[a]	1	4.033	2.846	0.520	0	9
	2	0.067	0.254	0.046	0	1
	3	7.967	1.426	0.260	4	12
	4	6.067	2.434	0.444	0	9
Baseline *DI*[b]	1	31.133	5.178	0.945	20	45
	2	0.000	---	---	---	---
	3	96.067	18.781	3.429	59	159
	4	61.700	5.658	1.033	50	72
H_2O_2 *DI*[c]	1	30.567	4.207	0.768	22	39
	2	---	---	---	---	---
	3	103.200	26.106	4.766	78	171
	4	84.100	6.89	1.258	68	99

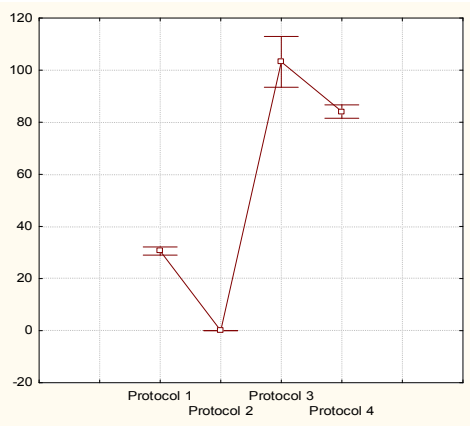
[a] x400; [b] DNA damage index; [c] H_2O_2 treated DNA damage index; [d] Standard Deviation; [e] Standard Error; [f] Minimum; [g] Maximum, Protocol 1: Tice and Vasquez, 1999; Protocol 2: Valverde et al., 1997; Protocol 3: Szeto et al., 2005; Protocol 4: Modified by authors



a) Nucleoids per field (x400)



b) Baseline DNA damage index



c) H_2O_2 treated DNA damage index

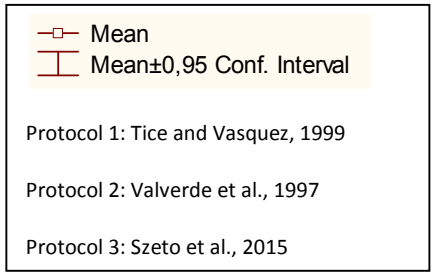


Fig. 1. Statistical comparison of parameters evaluated in the four protocols

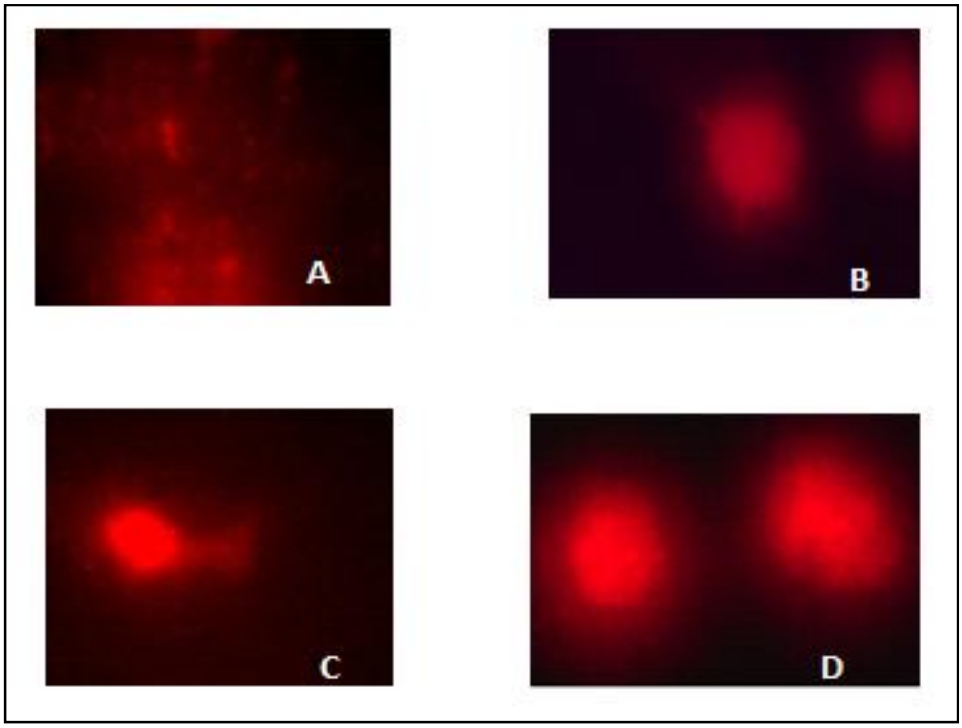


Fig. 2. Examples of comets with different degrees of DNA damage
2A. Disintegrated nucleoid. DNA is dispersed and scarce because of its small size.
2B and 2C. Nucleoid with DNA leakage as a narrow string, due to an irregular lysis of cell membrane.
2D. Nucleoids uniformly stained with precise contours that means minor DNA damage

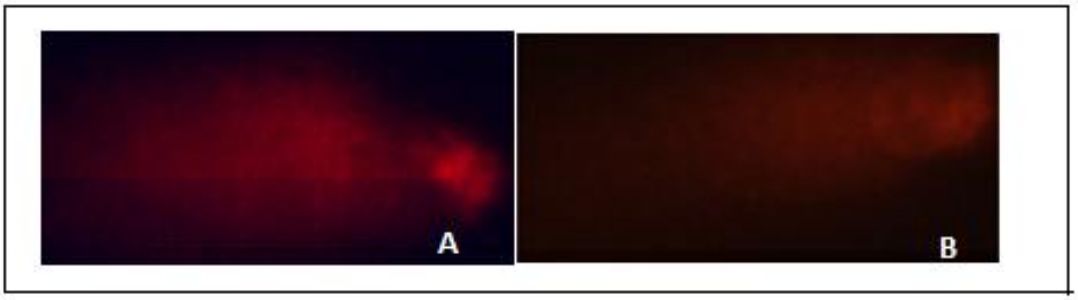
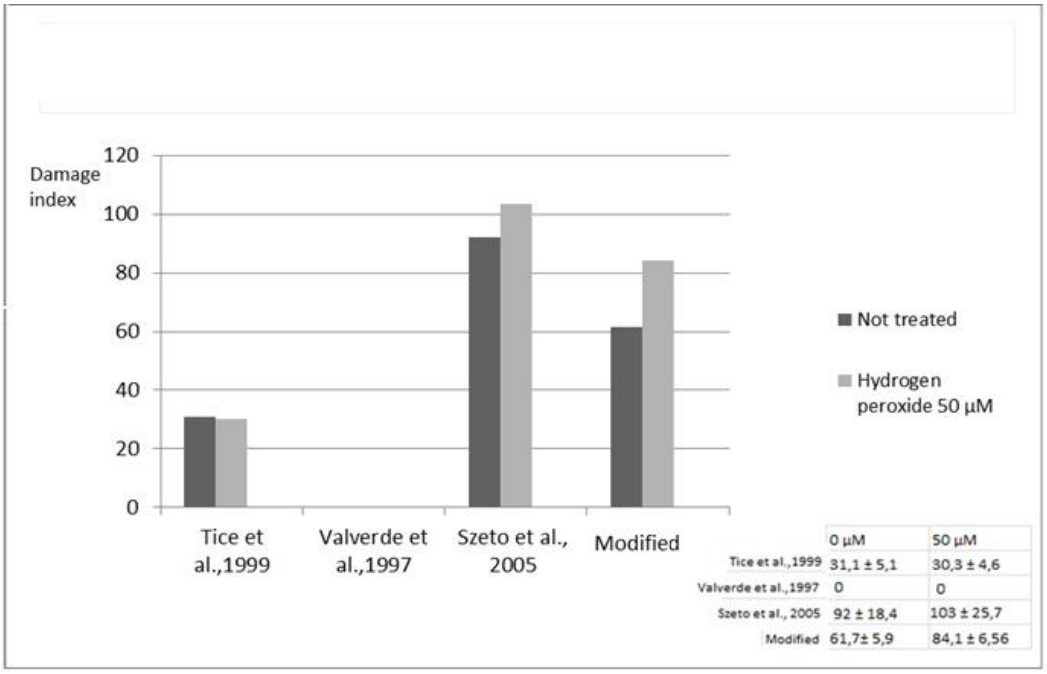


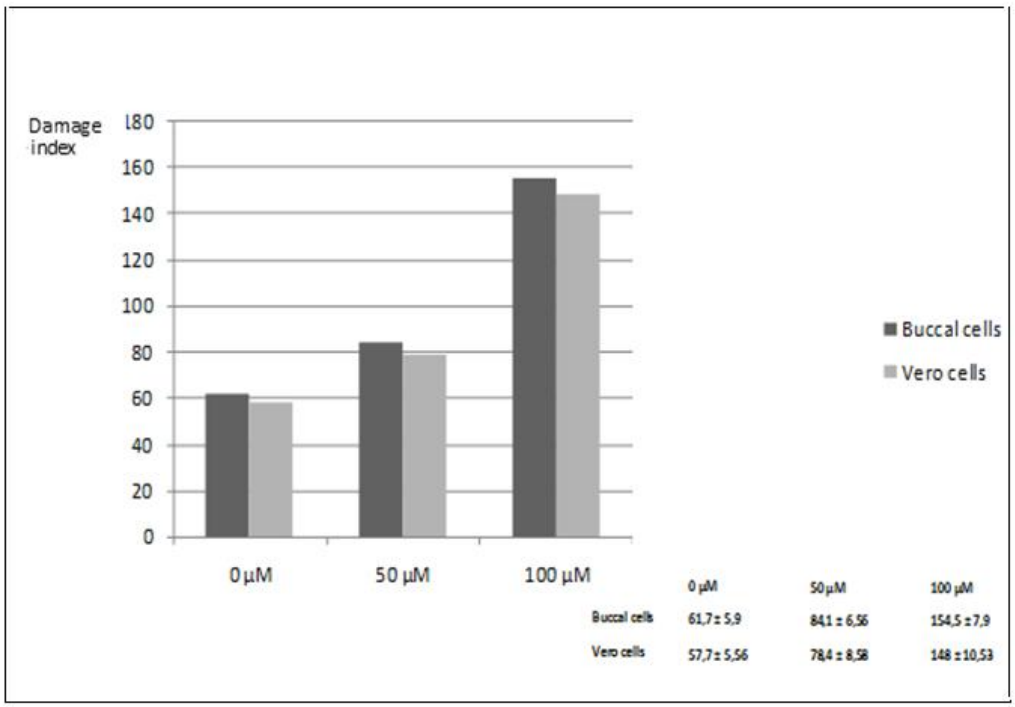
Fig. 3. Comet from buccal cell exposed to H_2O_2.
3A. DNA have migrated from the ¨head¨ to the ¨comet tail¨, that is longer and wider as DNA damage, is greater.
3B. Comet corresponding to level IV, where almost all DNA is into the tail

It is important to consider the mouth as a main entrance of the body and that it is lined with cells that may be the first to come in contact with toxic agents. It is also the place where several diseases such as lichen or alterations like periodontal disease and leukoplakia are developed and may contribute to increased DNA damage. That DNA damage isn´t associated with exposure to environmental genotoxicity, but linked to local inflammation [16,21]. It is worth noting that in recent years oral mucosal cells have been utilized in assessing DNA damage in human populations due to the easiness in obtaining the sample [6]; but there are certain technical aspects that need to be addressed in order to increase the application of this variant of the assay [4,12,13,22,23].



	0 µM	50 µM
Tice et al.,1999	31,1 ± 5,1	30,3 ± 4,6
Valverde et al.,1997	0	0
Szeto et al., 2005	92 ± 18,4	103 ± 25,7
Modified	61,7± 5,9	84,1 ± 6,56

Fig. 4. Comparison of damage index (*DI*) between buccal and Vero cells exposed to increasing concentrations of hydrogen peroxide (H_2O_2)



	0 µM	50 µM	100 µM
Buccal cells	61,7± 5,9	84,1 ± 6,56	154,5 ± 7,9
Vero cells	57,7± 5,56	78,4 ± 8,58	148 ± 10,53

Fig. 5. Comparison of Index of Damage between buccal and Vero cells exposed to increasing concentrations of hydrogen peroxide

A review of several protocols for the Comet assay led us to compare three of them and also to develop a fourth one. The protocol developed by our team resulted from modifications at several critical points. In the protocol of Szeto et al. (number 3) [13], there is a majority of

absolutely round nucleoids alternating with unviable ones, in which the DNA escapes as narrow strings, that may be the consequence of irregular lysis, where are zones in which membrane disruption is absolute and the proteins are stripped, interspersed with others where the membrane remains intact. These unusable nucleoids are interpreted as the result of an inefficient digestion that did not allow for the disassembly of membrane proteins, which includes intercellular complexes, resulting in membrane bands that remained as obstacles for DNA migration out of the nucleoid, which seems to be "stepwise and cooperative" [24].

Moreover, it has been shown that an irregular protein digestion could hinder migration [25] by allowing the formation of large loops in certain sites interfering with the relaxation of supercoiled DNA [26]. This phenomenon could affect the relationship between the relaxation of the supercoiled DNA and the amount of breaks present in the molecule. An insufficient protein digestion is related to the differences in the generation and migration of the DNA loops [27].

The variations introduced by our group in the established protocols are related to several critical points and will be explained below, focusing on each step of the procedures that were modified and their significance in the optimization of the Comet assay in buccal cells.

4.1 Buccal Cell Sampling

Sample collection started with a 2 minutes vigorous mouthwash, in order to remove the already detached cells from non-keratinized epithelium [28]. In a healthy mouth, a large proportion of cells obtained during sampling may be leukocytes [29]. We looked for an intermediate option that could guarantee an adequate shear force on the buccal mucosa, in order to obtain enough cells without damage that could introduce blood cells in the sample. Our option was the use of the Ayre spatula that has a bilobed contour at the scraping end, while at the grip end, the contour takes a more open angle which is more suitable for the inside of the cheeks. That's why the spatula was reversed and the usual grip area was used to scrap the inner lining of the cheeks. The number of viable cells increased approximately 10% relative to that obtained with brushing, and no bleeding was detected. The higher amount of living epithelial cells obtained with our approach could contribute to decrease the number of comet “clouds" related to death cells that usually appear in high number when the buccal cells are sampled [30].

4.2 Application of Modified Protocol to Vero Cells

It was considered necessary to evaluate if a greater DNA damage was artefactual [31]. Comet assay was performed on Vero cells that were treated in two alternative ways: after protocol 1 [14] and protocol 4. With protocol 4, DNA damage index in Vero cells was higher but not significantly than that observed in the same cells processed according to protocol 1.

The DNA damage index in Vero cells was higher than that of the buccal cells, but difference also wasn´t significant. The lack of significant differences in the DNA damage indexes between both cell types could be interpreted in the sense that our protocol (in spite of the double digestion) did not introduce a significant increase in DNA damage.

4.3 Enzymatic Digestion

In Valverde et al. protocol 2 [12], the cells were digested with 10 mg/ml proteinase K for 60 minutes at 37°C, which proved to be excessive and resulted in a general nucleoid disintegration. In Protocol 3; Szeto et al. [13], digestion with 0.025% trypsin was performed in suspension using a volume of 300 µl in the first assay while in the second and third ones, by pouring 50 µl on the slide, both followed by 1 mg/ml of proteinase K digestion on the slide. The conditions in the last two assays proved to be better and sustained a sufficient number of nucleoids so as to assess DNA damage although with a tendency to underestimate it when compared to results with protocol number 4.

In our protocol (4^{th}), the digestion time was not modified, but the trypsin concentration was increased to 0.05%, followed by one hour (1 mg/ml) treatment with proteinase K also at 37°C, pH 7.4.

The protocol described by Szeto et al. [13] was modified by us not only by increasing trypsin concentration, but also by performing the digestion in a 1.5 ml eppendorf tube before embedding the cells in the agarose. The arrangement of the cells for the digestion and duplication of the trypsin concentration were

crucial to improve results. In our protocol, the volume of trypsin digestion solution (buffer plus enzyme) varied from 50 µl when poured onto slides (protocol 3) to 200 µl with cells in suspension, while the volume of proteinase K digestion was increased from 100 (protocol 3) to 500 µl (protocol 4). When cells were digested in suspension at the same temperature and time as that of Szeto et al. [13] protocol, the yield of uniform nucleoids increased considerably.

The irregular contours of the nucleoids may be the result of incomplete digestion when cells already embedded in agarose are treated with proteases. There are reports that digestions have been shown to be incomplete due to an entrapment in a narrow network of barriers, so that the direct and uniform interaction between the enzymes and the extracellular matrix is impeded. These barriers may be sufficiently narrow to hinder the diffusion of the enzymes but also large enough to allow access to the substrates in other areas [32,33]. This could result in nucleoids with leakage areas alternating with others without migration due to a deficient membrane lysis. According to these evidences it was considered that changing the digestion site and performing it with cells in suspension would lead to a better sequential enzymatic treatment, enabling a more uniform access of enzymes to epithelial substrates without interference of agarose networks [32,33].

4.4 Alkaline Lysis and Unwinding Conditions

Longer lysis time in high salinity deproteinizing solution favors DNA migration [34]. Our lysis incubation conditions for seven days contributed to enhance strand damage expression and the sensitivity of the test. In addition, DNA migration into the comet tail also increases as the unwinding time develops [35]. The effect of a longer unwinding time on DNA migration is related to the expression of a greater amount of alkali-labile sites that become single-strand breaks and allows for greater relaxation of DNA loops [34,36]. The 20% increase in unwinding time to 25 minutes improved both the sensitivity and responsiveness of our protocol.

Another critical point regarding migration and comets integrity is pH [37]. In our protocol the pH was kept at 9.1. This pH conditions were enough to reveal increasing levels of single strand breaks, a fact that increases sensitivity when cells were treated with hydrogen peroxide.

4.5 Electrophoresis Temperature

The electrophoresis temperature, a critical parameter that if is raised, increases DNA migration [36,38], was strictly maintained at 20°C; under our conditions after 20 minutes running, the buffer temperature was only increased by 1.3°C on average. This temperature has been reported to be adequate for the expression of DNA breaks [39], but through limiting its elevation during electrophoresis, migration does not exceed to the point where nucleoids are no longer viable.

5. CONCLUSIONS

The protocol proposed here represents an improvement of the comet assay technique in mucosa buccal cells that is expressed as a higher proportion of USCN and a good response to oxidative treatment. The critical features of our protocol are: i) the use of an inverted Ayre spatula to obtain a higher amount of living non-keratinized epithelial cells of the oral mucosa; ii) the double digestion *with cells in suspension* prior to the imbibition in agarose results in a more uniform and efficient digestion; iii) the increased digestion volume allows an adequate DNA access for enzymes and reduces considerably the interference by proteins resulting in a better DNA migration into the comet tail; iv) a seven-day period of incubation that enhances alkaline lysis; as well as a 25 minutes of unwinding at pH 9.1; v) the strict control of temperature at 20°C during the electrophoretic run.

This work has been done as a contribution to the effort to standardize the less invasive Comet assay in buccal cells as a tool for genetic biomonitoring.

CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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