Original Research

An early and late cytotoxicity evaluation of lidocaine on human oral mucosa fibroblasts

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Abstract

Local anesthetic drugs are extensively used in dentistry. However, the cytotoxic effects of these pharmaceutical compounds remain unclear. In this work, we have evaluated the cell viability and cell function of human oral mucosa fibroblasts exposed to different concentrations of lidocaine for increasing incubation times, using a global screening methods including structural, metabolic and microanalytical analyses. Our results demonstrate that lidocaine is able to alter cell viability and function even at low concentrations and times, although the effect of lidocaine concentration was more important than the incubation time. First, the structural analysis methods revealed that $\geq$5% concentrations of lidocaine are able to significantly reduce cell viability. Then, the metabolic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and water-soluble tetrazolium salt (WST-1) assays suggest that concentrations starting from 1% were able to significantly hinder cell physiology. Finally, electron-probe X-ray microanalysis confirmed the deleterious effects of lidocaine and allowed us to demonstrate that these effects are associated to an apoptosis process of cell death. Therefore, care should be taken when lidocaine is clinically used, and the lowest efficient concentrations should always be used. Furthermore, these results suggest that the comprehensive evaluation method used in this work is accurate and efficient for screening of local anesthetics.

Keywords: Lidocaine, human oral mucosa fibroblasts, MTT, WST-1, X-ray microanalysis, calcein ethidium

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Introduction

The use of local anesthetics has been of great demand in numerous dental procedures. Nowadays, one of the most commonly used local anesthetics in dentistry is lidocaine.[1] A reversible anesthetic effect and a wide margin of safety when administered in proper doses to obtain anesthesia are hallmarks of this drug class. However, some studies suggest that certain anesthetics applied locally can increase the risk of oral paresthesia[2] or can cause muscular contraction and stiffness.[3] All these findings suggest that local anesthetics could have some cytotoxic effects and point out the need of developing new wide-ranging methodologies to evaluate the cytotoxicity of these components.

In this milieu, numerous studies have been developed with the aim of studying the cellular effects of local anesthetics by using distinct cells types. Some of these studies demonstrated that local anesthetics, including lidocaine, are able to induce conformation changes on the cytoskeleton of erythrocytes[4] and fibroblasts,[5] cell proliferation alterations of cornea epithelial cells[6] and lung fibroblasts[7] and cell viability alterations on human articular chondrocytes,[8] lymphoma cells,[9] neuroblastoma cells,[10] Schwann cells and neurons.[11] Similarly, Grouselle *et al.*[12] showed that highly liposoluble local anesthetics can reach the mitochondria and modify the transmembrane potential of 3T3 fibroblasts, and recent studies performed by Villarruel *et al.*[13] demonstrated that lidocaine is able to stimulate apoptosis on human gingival fibroblasts. Most of these reports focused on specific aspects of the cytotoxic effects of lidocaine, and very few works made use of a highly-sensitive and accurate combination of methods and techniques for an efficient evaluation of this drug.

Different approaches have been used for the determination of cell viability of cultured cells incubated in the presence of drugs or other cytotoxic compounds. Several methods focused on the detection of permeability alterations in the cell membrane such as trypan blue.[14] However, this technique is not accurate enough to predict early cell damage, and it is able to identify cell alterations only once they have become irreversible. Other methods are

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based on the quantification of all major intracellular cell elements, especially potassium and sodium[15–17] by means of electron-probe X-ray microanalysis (EPXMA). This accurate and highly-sensitive technique makes possible to simultaneously analyze the intracellular ionic concentrations and the ultrastructure of the cells.[18] By using EPXMA, our group has previously quantified the intracellular ionic composition of different cell types, including U937,[16] primary cell cultures of human oral mucosa keratinocytes,[19] umbilical cord endothelial cells,[20] Wharton's jelly stem cells,[21] rabbit cornea endothelial cells[22] and human dental pulp stem cells.[23] Other types of methods are based on the release on intracellular cell components such as DNA, whose amount in the cell culture correlates with the number of dead cells.[24] In addition, several highly-sensitive metabolic methods can be used to evaluate not only the viability of the cells, but also the cell proliferation rate and the physiological status of the cells by analyzing cell function at the cytoplasmic and mitochondrial levels using calcein and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)/WST-1 reagents.[21,24–26]

As shown above, several studies have previously evaluated the cytotoxicity of different local anesthetics, and it is well known that lidocaine is a cytotoxic agent exhibiting cytotoxicity to many cells *in vitro*. However, most of these studies used one or a reduced number of methods, and very few works used a combined comprehensive approach including several techniques of different nature. In addition, most of these studies have been developed on extra-oral cell sources and on specific short ranges of dosages and times. The aim of the present study was to develop a new screening method to evaluate the cytotoxic effects of local anesthetics using an efficient combination of methods and techniques, using lidocaine as a model. In this regard, we determined the cell viability of human oral mucosa fibroblasts (HOMF) exposed to an ample range of concentrations of lidocaine for different incubation times using a novel comprehensive approach that included five different methods to evaluate several crucial parameters: (1) DNA quantification to analyze cell membrane integrity; (2) WST-1 to determine mitochondrial metabolic function and cell proliferation; (3) MTT to analyze mitochondrial metabolism; (4) calcein/AM-ethidium homodimer-1 assays to determine cell membrane integrity and cytoplasmic metabolic function; (5) EPXMA to quantify the concentrations of all major intracellular elements. By using this combinatorial approach for the first time, we increased the accuracy of the results to obtain a global view of the main processes involved in cell death after lidocaine exposure that may have clinical implications.

Materials and methods

Human oral mucosa biopsies

A 3×3 mm human oral mucosa biopsies were obtained from five healthy donors subjected to minor oral surgery procedures (third molar extraction). The average age of the donors was 44.6 years. These samples were washed in Dulbecco's modified Eagles's minimal essential medium (DMEM; Sigma-Aldrich, Steinheim, Germany) with 6% antibiotics/antimycotics solution (10,000 units/mL of penicillin, 10 mg/mL of streptomycin and 25 µg/mL of amphotericin B; Sigma-Aldrich) and processed within 6 h. All patients gave their consent to participate in the study. This study was approved by the local research and ethics committee.

Isolation and culture of human oral mucosa fibroblasts

All cells were isolated and cultured as previously reported.[19] Briefly, a 2% *Clostridum hystoliticum* type I collagenase solution was used at 37°C for 10–12 h to digest the tissue extracellular matrix, and cells were harvested by centrifugation at 1000 rpm for 10 min. DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics/antimycotics was used as culture medium. Cells were subcultured using trypsin 0.5 g/L ethylenediaminetetraacetic acid 0.2 g/L solution (Sigma Aldrich). All cells used in this study corresponded to the first five cell passages.

Preparation of lidocaine dilutions and cell culture assays

In this study, we used commercially available 2% lidocaine HCl with epinephrine 1:100,000 (Octocaine™, Clarben Laboratory, Madrid, Spain). This local anesthetic was diluted in DMEM without antibiotics at a final concentration of 0.05% (0.00004 mol/L), 0.5% (0.00043 mol/L), 1% (0.00085 mol/L), 5% (0.00427 mol/L), 10% (0.00853 mol/L), 25% (0.02134 mol/L), 50% (0.04267 mol/L), 75% (0.06401 mol/L) and 100% (0.08535 mol/L) of the original commercially available presentation, and HOMF were incubated in all these lidocaine concentrations for 30 min, 1, 6, 12, 24 and 48 h. As positive control, HOMF were cultured with DMEM and as a negative control, HOMF were previously treated with 2% triton X-100 detergent (Sigma, St. Louis, MO). Triton X-100 is a chemical compound that has been previously described to produce high cytotoxic effects and it has been used as negative control in previous works.[27]

Determination of cell viability and cell function at the structural, metabolic and microanalytical levels

In this work, we analyzed HOMF to determine both the vital status of the cells (i.e. live vs. dead cells) and their functionality (functional vs. dysfunctional cells) using a combination of screening methods. To determine cell viability at the structural level, DNA quantification and ethidium homodimer-1 staining methods were used. To analyze cell function at the metabolic level, WST-1, MTT and calcein/AM methods were used. To determine cell viability and specific cell death mechanisms at the microanalytical level, EPXMA was used.

DNA quantification. In order to quantify the cell DNA released to the culture medium as a consequence of cell death, 10 µL supernatant aliquots were taken from each experimental group. Each aliquot was diluted in distilled nuclease-free water (Ambion-Life Technologies,

Austin, TX) until a final volume of 100 μL and free DNA quantified by using a SmartSpec™ plus Spectrophotometer (BIO-RAD, Hercules, CA) at 260–280 nm wave-lengths. Six different measures were taken from each sample corresponding to each specific condition (concentration and incubation time).

Cell proliferation and function assays at the mitochondrial level (WST-1 and MTT). In order to evaluate metabolic cell function and cell proliferation capability, we used WST-1 proliferation reagent (Roche, Germany) and MTT Cell proliferation kit (Roche) assays. Briefly, 10^4 cells were seeded per well in a 96-well plate with 100 μL of DMEM culture medium. After 48 h, cells were exposed to the different lidocaine concentrations for different periods. After each incubation time, the supernatant was removed and the cells were washed twice with phosphate-buffered saline. To quantify the cell proliferation, medium containing 1/10 volume of cell proliferation reagent WST-1 was added, the cells were incubated for 4 h at 37°C, and absorbance was quantified at 450–690 nm in a spectrophotometer. To quantify the cell metabolic activity, medium containing 1/10 volume of MTT labeling reagent was added, and the cells were incubated for 4 h at 37°C. Then, solubilization buffer was added and the cells were incubated overnight. Finally, absorbance was quantified at 550–690 nm in a spectrophotometer. For each specific condition (concentration and incubation time), six different measures were taken from each sample.

Cell viability and cell function determination by a dual cytoplasmic and nuclear calcein/AM-ethidium homodimer-1 (LIVE/DEAD™ assay). To simultaneously analyze cell function – as determined by cytoplasmic metabolism – and cell viability – as determined by cell membrane integrity – calcein/AM-ethidium homodimer-1 Viability/Cytotoxicity assay kit (Life Technologies, Carlsbad, CA) was used. For this assay, 2×10^4 cells were seeded per well in a chamber slide system (Lab-Tek II, Nunc, Denmark) with culture medium. After 48 h, HOMF were exposed to specific anesthetic concentrations for different periods of time. In this case, cells were incubated in 0%, 1%, 5%, 10%, 20%, 30%, 40%, 50%, 75% and 100% dilutions of lidocaine, and in the DNA TD_{50}, WST-1 TD_{50}, MTT TD_{50} specific concentrations previously determined by the other methods (see below). LIVE/DEAD™ assay was performed by following the manufacturer's recommendations and the cells were immediately analyzed using a fluorescence microscope (Nikon Eclipse 90i, Nikon, Japan). For each experimental condition, six images were taken at random areas of the slide. Then, each image was analyzed using the ImageJ software (MacBiophotonics, Ontario, Canada) developed at McMaster University in order to quantify the number of red (dead) and green (live) cells as previously reported.[23] For each condition, an average of 1000 cells were analyzed using this system.

Cell viability and cell function determination at the micro-analytical level EPXMA. For EPXMA, HOMF were subcultured on gold grids previously covered with a thin layer of Pioloform (polyvinyl butyral; Ted Pella, Inc., Redding, CA) following previously published methods[21,22] and sterilized overnight under UV light. Once the cells reached 70% confluence, HOMF were exposed to different lidocaine concentrations (DNA TD_{50}, MTT TD_{50}, WST-1 TD_{50}, see below) for different time periods (30 min, 1, 6, 12, 24, 48 h). After the anesthetic exposition, gold grids containing the cultured cells were washed with ice-cold distilled water for 5 s to remove the extracellular medium and immediately plunge-frozen in liquid nitrogen. To determine the total intracellular ion content, we used the peak-to-local-background (P/B) ratio method.[21,22,28] For each specific condition (concentration and incubation time), 20 cells were analyzed.

Statistical analysis and toxic dosage 50 (TD_{50}) determination

In this study, we determined the TD_{50} as the local anesthetic concentration able to alter 50% of the evaluated cells population. The results obtained for each assay (quantification of released DNA, WST-1 and MTT) were used to calculate the TD_{50} separately for each incubation time using a regression line formula. Once we determined the TD_{50}, LIVE/DEAD™ assay and EPXMA were carried out on specific concentrations.

To compare the results obtained for each sample (i.e. each concentration and each incubation time) with the results corresponding to the positive control, we used the Mann-Whitney (MW) test. This test was also used to identify statistical differences between the highest and the lowest lidocaine concentrations. To evaluate the influence of the period of time and the concentration on the cells incubated in lidocaine, we used the Kruskal-Wallis (KW) test. Kendall Tau (KT) test was used to correlate the cell viability or cell function results obtained for different concentrations and times.

To determine the effect of lidocaine in relation to live, functional cells, all results were normalized using the positive and negative controls as references as previously reported.[27] In short, for each concentration, time and technique, the values obtained for the positive control (live cells) were considered as 100% for the WST, MTT and LIVE/DEAD™ assays, whereas the values obtained for the negative control were considered as 0% for the same methods. For DNA release quantification, positive controls were considered 0% and negative controls as 100%. Then, all values corresponding to the different assays carried out here were normalized to the positive and negative controls and expressed as percentage of viability or cell function in relation to the control.

Results

Determination of cell viability by released DNA quantification

The cell viability analysis as determined by DNA quantification indicated that the amount of DNA released to the culture medium increased with the exposition period

(KW $P < 0.01$ for all lidocaine concentrations) and specially the anesthetic concentration (KW $P = 0.000$ for all times, Table 1, Figure 1). The statistical analysis revealed that the released DNA was significantly higher when high lidocaine concentrations were used as compared with the lower lidocaine concentrations (MW $P < 0.001$) for all samples, and that most high lidocaine concentrations significantly decreased cell viability as compared to positive control cells (Figure 1). Strikingly, a statistically significant correlation was found between DNA release and the concentration of lidocaine in the culture medium for all times (KT $P = 0.000$ and r ranging between 0.366 and 0.237). The correlation with time in culture was significant only for specific lidocaine concentrations (Table 2). Interestingly, the use of 75% lidocaine concentrations resulted in 100% DNA release for some incubation times. In this study, once the viability analysis was performed by using DNA quantification, we determined the TD_{50} values for each exposition time. As shown in Table 3, our results showed that the TD_{50} was 28.52% for 30 min, 24.60% for 1 h, 24.15% for 6 h, 24.15% for 12 h, 23.51% for 24 h, 22.20% for 48 h of lidocaine exposition and 24.52% for all times (mean TD_{50}).

Table 1 Kruskal-Wallis (KW) statistical test for the comparison of the lidocaine concentration ([]) and the exposure time (T) for the DNA, WST-1, MTT and LIVE/DEAD™ assays.[a]

		DNA	WST-1	MTT	Live/dead
	30 min	0.000	0.000	0.000	0.0000
	1 h	0.000	0.000	0.000	0.0000
[]	6 h	0.000	0.000	0.000	0.0000
	12 h	0.000	0.000	0.000	0.0000
	24 h	0.000	0.000	0.000	0.0000
	48 h	0.000	0.000	0.000	0.0000
	PC	0.000	0.001	0.000	0.0003
	0.05%	0.000	0.024	0.001	
	0.5%	0.000	0.030	0.001	
	1%	0.001	0.006	0.001	0.6028
T	5%	0.000	0.000	0.000	0.0001
	10%	0.000	0.000	0.000	0.0000
	25%	0.000	0.000	0.000	
	50%	0.001	0.000	0.000	0.3948
	75%	0.000	0.000	0.000	1.0000
	100%	0.000	0.000	0.000	1.0000

[a]The statistical P values are shown for each comparison.

Cell proliferation and cell function assays by WST-1 and MTT determination

The cell proliferation and functional studies using the metabolic techniques WST-1 and MTT demonstrated that the metabolic activity varied among the different study groups. As shown in Figure 2, the WST-1 and MTT cell activity tended to decrease in response to increasing incubation times (KW $P < 0.01$ for most of the lidocaine concentrations, Table 1) and to increasing lidocaine concentrations (KW = 0.000 for all times for both methods, Table 1).

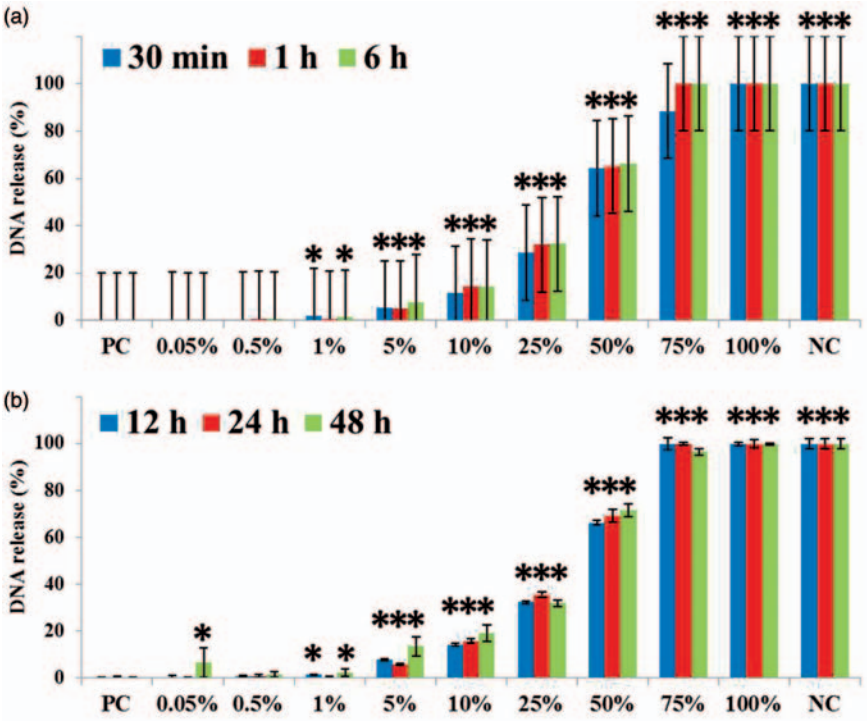

Figure 1 DNA release quantification of HOMF exposed to lidocaine. (a) Mean DNA quantification results obtained for HOMF incubated in increasing lidocaine concentrations for short time periods (30 min, 1 h and 6 h). (b) Mean DNA quantification results obtained for HOMF incubated in increasing lidocaine concentrations for long time periods (12, 24 and 48 h). All results are normalized to a maximum DNA release of 100% and a minimum release of 0% using the positive control (PC) and the negative control (NC). Standard deviation is shown by error bars. These results correspond to six different measures taken from each sample. Statistically significant differences in comparison with the control group (PC) are labeled with asterisks (*). (A color version of this figure is available in the online journal)

Table 2 Kendall Tau (KT) correlation test for the comparison of the lidocaine concentration ([]) and the exposure time (T) for the DNA, WST-1, MTT and LIVE/DEAD™ assays.[a]

		DNA		WST-1		MTT		Live/dead	
		r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
[]	30 min	0.935	0.000	−0.631	0.000	−0.685	0.000	−0.7278	0.0000
	1 h	0.916	0.000	−0.547	0.000	−0.631	0.000	−0.7380	0.0000
	6 h	0.962	0.000	−0.545	0.000	−0.610	0.000	−0.6964	0.0000
	12 h	0.919	0.000	−0.487	0.000	−0.626	0.000	−0.7069	0.0000
	24 h	0.885	0.000	−0.622	0.000	−0.626	0.000	−0.6219	0.0000
	48 h	0.866	0.000	−0.295	0.004	−0.639	0.000	−0.5340	0.0000
T	PC	0.366	0.008	0.526	0.000	0.067	0.624	−0.1200	0.3326
	0.05%	0.641	0.000	0.382	0.005	0.250	0.067		
	0.50%	0.686	0.000	0.631	0.000	0.255	0.062		
	1%	0.416	0.002	0.506	0.000	0.191	0.163	0.0411	0.7398
	5%	0.731	0.000	0.364	0.008	−0.448	0.001	−0.4221	0.0007
	10%	0.885	0.000	−0.134	0.327	−0.393	0.004	−0.6945	0.0000
	25%	0.396	0.004	−0.266	0.052	−0.342	0.012		
	50%	0.591	0.000	−0.209	0.128	−0.238	0.082	−0.0920	0.5202
	75%	0.142	0.301	−0.244	0.075	−0.231	0.092	−0.2182	0.1432
	100%	0.237	0.085	−0.441	0.001	−0.725	0.000	−0.2182	0.1432

[a]The statistical *P* values and the correlation *r* coefficient are shown for each comparison.

Table 3 Toxic dosage 50 (TD_{50}) of lidocaine as determined by DNA quantification and WST-1 and MTT metabolic assays at each incubation time

	DNA	WST-1	MTT
30 min	28.52%	5.45%	3.56%
1 h	24.60%	5.85%	7.48%
6 h	24.15%	4.75%	9.14%
12 h	24.15%	3.40%	2.89%
24 h	23.51%	1.12%	6.23%
48 h	22.20%	4.71%	5.21%

The MW statistical comparison of WST-1 and MTT-determined cell function results with positive controls demonstrated a significant mitochondrial function decrease for most lidocaine concentrations ≥5%, suggesting that concentration had a deeper effect on cell function than incubation time (Figure 2). In fact, using WST-1 assay, we found that the cell proliferation rate was significantly lower in the group treated with the highest lidocaine concentrations than in the group incubated in the lowest concentrations (MW $P < 0.001$), and the same phenomenon was found for MTT results. A statistically significant correlation was found between WST-1 or MTT metabolic activity and the concentration of anesthetic in the culture medium for all times (KT $P = 0.01$ and r ranging between 0.067 and −0.725 for MTT; and between 0.526 to −0.441 for WST-1). The correlation with time in culture was significant only for specific lidocaine concentrations (Table 2). The use of lidocaine concentrations as low as 5% for WST-1 and 10% for MTT were able to completely alter the mitochondrial function to 0% functional activity at long incubation times. Using the cell proliferation WST-1 assay, we observed that the TD_{50} (Table 3) was 5.45% for 30 min, 5.85% for 1 h, 4.75% for 6 h, 3.40% for 12 h, 1.12% for 24 h, 4.71% for 48 h of lidocaine exposition and 4.21% for all times (mean TD_{50}). For the MTT assay (Table 3), we detected that the TD_{50} was 3.56% for 30 min, 7.48% for 1 h, 9.14% for 6 h, 2.89% for 12 h, 6.23% for 24 h, 5.21% for 48 h of lidocaine exposition and 5.75% for all times (mean TD_{50}).

Cell viability and cell function analysis as determined by LIVE/DEAD™ assay

The use of the dual staining method LIVE/DEAD™ on cells incubated with different lidocaine concentrations revealed that the percentage of viable and functional cells tended to decrease with time in culture only for some specific dilutions (KW $P < 0.01$ for 5%, 10% and 20% of the lidocaine concentrations, Table 1) and especially, with increasing anesthetic concentrations (KW = 0.000 for all times, Table 1). The MW comparison with the positive control confirmed that the effect of the concentration on cell viability and cell function was more profound than the culture time (Figure 3). In fact, a significant negative correlation was found between cell viability and the lidocaine concentration for all times (KT $P = 0.000$ with r ranging between −0.5340 and −0.7380; Table 2). However, the correlation with the time in culture was significant only for concentrations ranging between 5% and 30% (Table 2).

As shown in Figures 3 and 4, incubation of the cells in lidocaine TD_{50} concentrations resulted in variable percentages of viable and functional cells, and a significantly higher toxicity and lower cell function was found at the

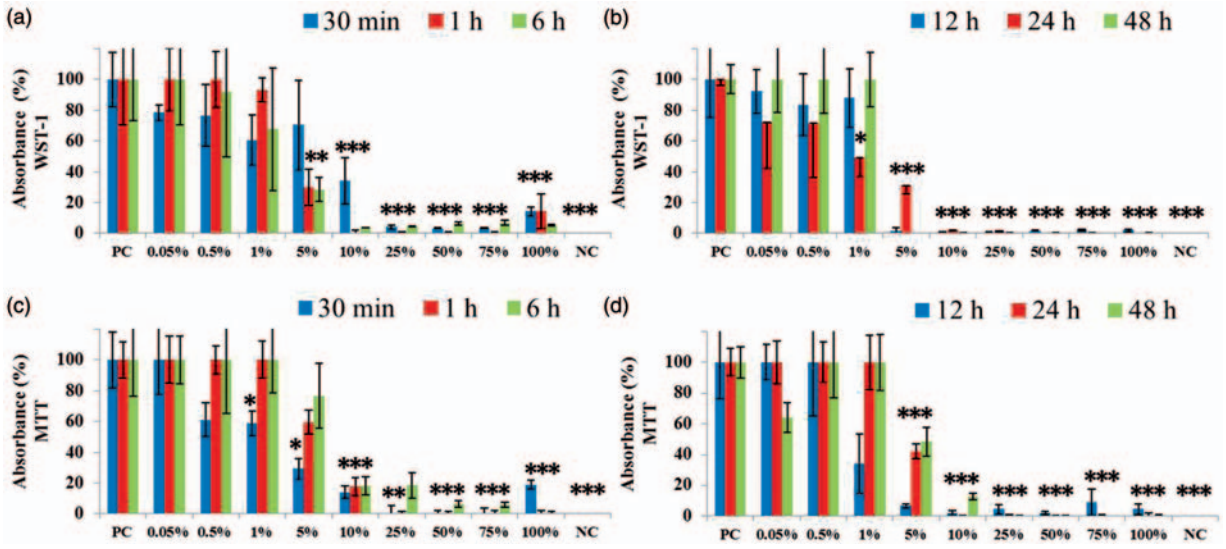

Figure 2 Metabolic activity determination of HOMF exposed to lidocaine. (a) Mean WST-1 quantification results obtained for HOMF incubated in increasing lidocaine concentrations for short time periods (30 min, 1 h and 6 h). (b) Mean WST-1 quantification results obtained for HOMF incubated in increasing lidocaine concentrations for long time periods (12, 24 and 48 h). (c) Mean MTT quantification results obtained for HOMF incubated in increasing lidocaine concentrations for short time periods (30 min, 1 h and 6 h). (d) Mean MTT quantification results obtained for HOMF incubated in increasing lidocaine concentrations for long time periods (12, 24 and 48 h). All results are normalized to a maximum value of 100% and a minimum value of 0% using the positive control (PC) and the negative control (NC). Standard deviation is shown by error bars. These results correspond to six different measures taken from each sample. Statistically significant differences in comparison with the control group (PC) are labeled with asterisks (*). (A color version of this figure is available in the online journal)

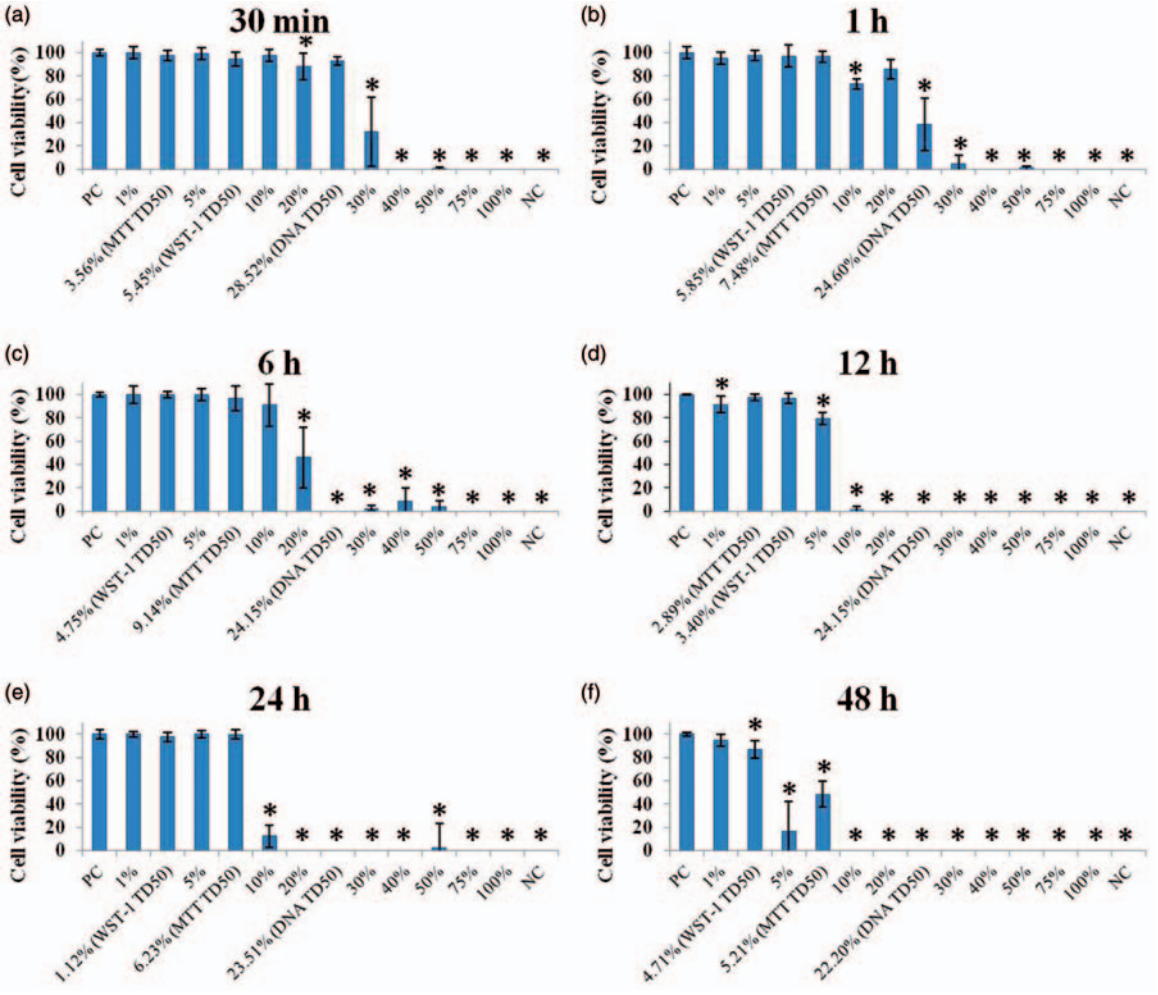

Figure 3 Cell viability and cell function analysis of HOMF exposed to lidocaine as determined by LIVE/DEAD™ assay. (a) Analysis at 30 min, (b) 1 h, (c) 6 h, (d) 12 h, (e) 24 h and (f) 48 h. PC: positive control, NC: negative control. Standard deviation is shown by error bars. These results correspond to six different measures taken from each sample. Statistically significant differences in comparison with the control group (PC) are labeled with asterisks (*). (A color version of this figure is available in the online journal)

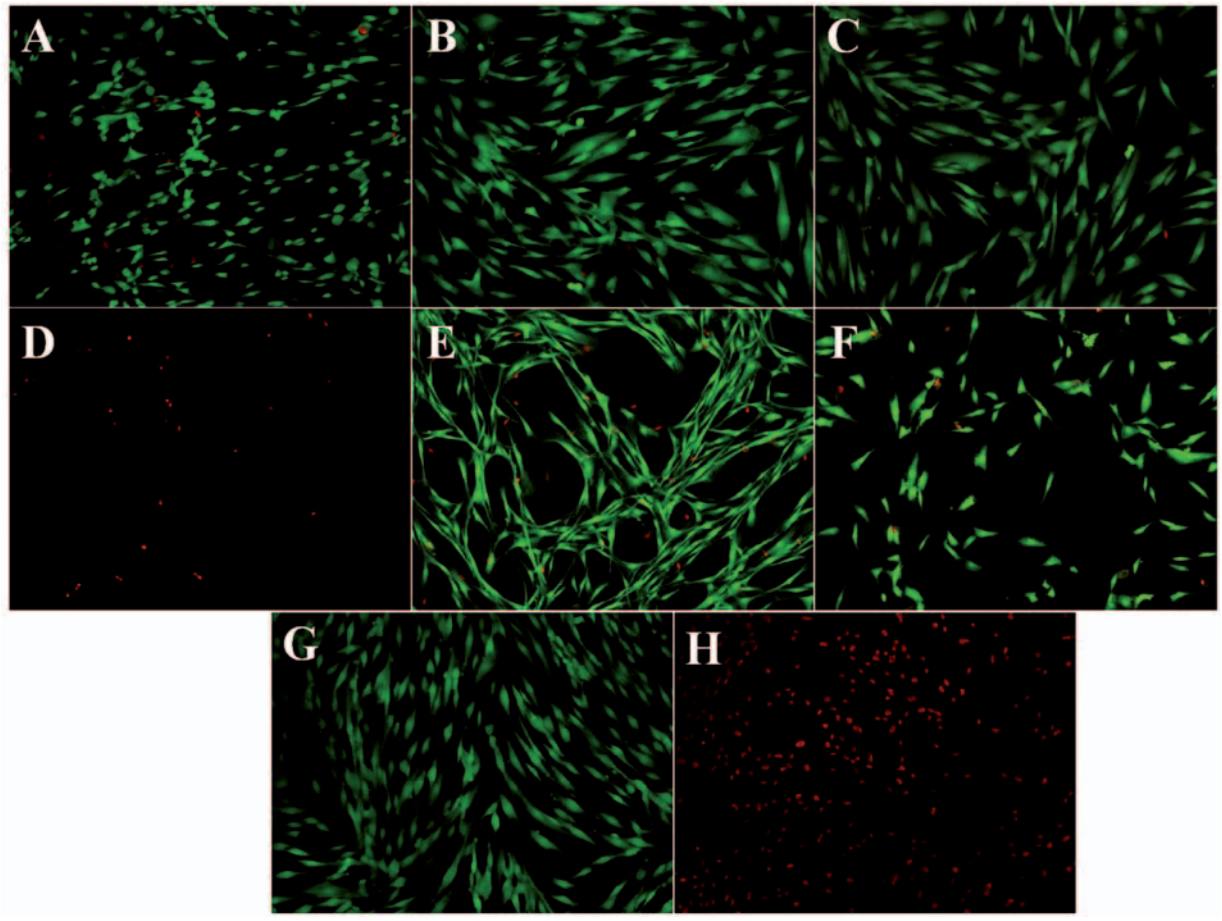

Figure 4 Illustrative images of HOMF exposed to lidocaine and analyzed by using LIVE/DEAD™ assays. Green cells correspond to live cells whereas dead cells are stained in red. Images corresponding to short (30 min) and long (24 h) periods of time are shown. (A) DNA TD_{50} 30 min, (B) WST-1 TD_{50} 30 min, (C) MTT TD_{50} 30 min, (D) DNA TD_{50} 24 h, (E) WST-1 TD_{50} 24 h, (F) MTT TD_{50} 24 h, (G) positive control and (H) negative control. These results correspond to six different measures taken from each sample. (A color version of this figure is available in the online journal)

longer incubation times. In addition, the use of lidocaine at the TD_{50} concentrations as determined by DNA quantification assays, was always associated to lower cell viability and function levels as compared to TD_{50} concentrations as determined by WST-1 and MTT assays. Remarkably, the use of 40% dilutions of the anesthetic was able to reduce cell viability and function to 0% according to the LIVE/DEAD™ assay even with 30 min of incubation time, whereas 10% concentration reduced viability to 0% at 48 h. Finally, the cytotoxic effects of the highest lidocaine concentrations demonstrated to be significantly higher than those of the lowest concentrations (MW $P = 0.000$).

Cell viability analysis as determined by EPXMA

The use of highly-sensitive microanalysis techniques on cells incubated on the different TD_{50} concentrations revealed a remarkable reduction of the K/Na ratio associated with the presence of lidocaine in the culture medium (Figure 5 and Table 4). In short, we found that the K/Na ratio of the control cells incubated in DMEM culture medium without lidocaine ranged between 8.85 for 12 h and 7.56 at 6 h (mean 8.25). In contrast, the K/Na ratio decreased to a mean value of 0.42 (ranging from 3.96 to 0.10) when DNA TD_{50} concentrations were used, and to a mean value of 3.23 (4.51–0.03) and 2.66 (3.96–0.16) for WST-1 and MTT TD_{50} concentrations, respectively. In general, the lowest ratios corresponded to cells treated with DNA TD_{50} concentrations. All K/Na ratios corresponding to DNA TD_{50} and MTT TD_{50} concentrations and most WST-1 TD_{50} concentrations were significantly lower than that of the control cells (MW $P < 0.010$; Figure 5).

On the other hand, the analysis of the intracellular contents of Cl revealed that the use of DNA TD_{50} lidocaine concentrations was associated to a decrease of this intracellular ion after long incubation times (24 and 48 h), and a decreasing trend was also found when MTT TD_{50} were used. However, WST-1 TD_{50} concentrations tended to decrease at 24 h, but increased at 48 h. Other relevant intracellular ions such as P, Mg and S did not considerably vary with time in culture. Finally, the levels of Ca tended to increase with time only when WST-1 and MTT TD_{50} concentrations were used. However, the levels of Ca were higher than the control values when lidocaine was added to the culture medium for most concentrations and times, with some values increasing over 10-fold at the longest time periods (Table 4).

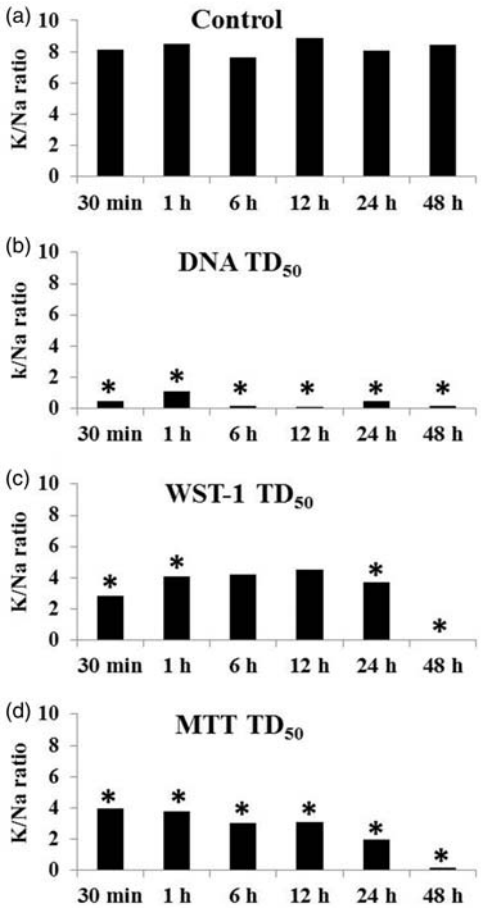

Figure 5 Cell viability and cell function analysis of HOMF exposed to lidocaine as determined by electron-probe X-ray microanalysis. Intracellular K/Na concentration ratios as determined by electron probe X-ray microanalysis (EPXMA) on cells incubated in different concentrations of lidocaine (a) 0% as control, (b) TD_{50} obtained for DNA, (c) TD_{50} obtained for WST-1 and (d) TD_{50} obtained for MTT, during increasing incubation times. These results correspond to the analysis of 20 independent cells per group. Statistically significant differences in comparison with the control group (PC) are labeled with asterisks (*)

Discussion

Local anesthetics are among the most used drugs in dentistry due to their capability to control pain. Lidocaine is widely used for temporomandibular joint treatments,[29] endodontic procedures[30] and other dental treatments.[31,32] However, local anesthetics may cause systemic[33,34] and local complications,[2,3,35,36] and few investigations have been carried out to elucidate the cytotoxic effects of lidocaine using a wide-ranging approach with several concentrations and incubation times. Most previously published works are based on one or few number of cell viability and cell function tests, such as MTT,[30] lactate dehydrogenase activity[8] and trypan blue exclusion tests.[37] For these reasons, development of comprehensive methods focused on the biological effects of this anesthetic on human oral cells is still in need.

The objective of the present work was to describe new screening methods for the characterization of local anesthetics, and to evaluate the cell toxicity and biological effects of different concentrations and incubation times of lidocaine on human oral fibroblasts using these methods. In general, our results evidenced that both the concentration and the incubation time affected cell viability and cell physiology. However, the concentration of lidocaine demonstrated to be more important than the incubation time. Previous studies demonstrated that lidocaine can induce apoptosis on T-cell line cultures in a time-dependent manner, as determined by annexin V assay, although different concentrations were not compared in that work.[38] Recently, another investigation described a cytotoxic effect of lidocaine on human chondrocytes in a dose- and time-dependent manner, although only short times were evaluated.[8] Our study confirms these previous works and shed light on the cytotoxic effects of lidocaine for a wide range of times and concentrations using highly-sensitive screening methods.

As expected, our results revealed that low concentrations of lidocaine were able to cause very few cell damage, and the results of the WST-1 and MTT metabolic assays evidenced that low concentrations of lidocaine were not able to significantly affect mitochondrial function. Indeed, the analysis of cells incubated in lidocaine concentrations up to 5% confirmed that a high percentage of cells had proper cytoplasmic metabolic function and membrane integrity as determined by LIVE/DEAD^{TM} and DNA release, and the metabolic activity of cells incubated in the lowest lidocaine concentrations did not significantly differ from control cells. Future studies are necessary to determine whether or not these mild cytotoxic effects induced by low dosages of lidocaine are reversible. On the other hand, the present study confirmed that high concentrations of lidocaine were associated to severe cytotoxic effects. In this regard, our results showed that incubation of the cells in high concentrations of lidocaine was associated to a cell membrane integrity alteration as demonstrated by the ethidium homodimer-1 test of the LIVE/DEAD^{TM} assay and DNA release. These results were confirmed by the mitochondrial assays WST-1 and MTT, which demonstrated a severe functional alteration in cells incubated in concentrations equal or higher than 10%. We speculate that lidocaine could induce structural cell damage at concentrations used clinically (100% lidocaine). Several publications previously demonstrated that local anesthetics can produce cell morphologic changes such as vacuolization of human skin fibroblasts.[39] Another report described that lidocaine, mepivacaine and prilocaine were able to produce cell rounding and detachment from the substrate of rat gingival fibroblasts, depending of anesthetic type, concentration and duration of exposure.[40] Lidocaine has been reported to cause, dose and time dependently, conformational change on red blood cell as result of spectrin aggregation.[4] Furthermore, a study performed on neuroblastoma cells revealed that local anesthetics, including lidocaine, induced apoptosis in a concentration-dependent manner, although the highest concentrations were able to induce necrotic cell death.[10] All these reports confirm the cytotoxic effects of lidocaine found in the present work.

Though recent investigations on the cytotoxicity of local anesthetics have evaluated the mechanism of cell death induced by these drugs in maxillofacial cells and tissues,[13,41] the cell death mechanism generated by lidocaine

Table 4 Intracellular concentrations of Na, Mg, P, S, Cl, K and Ca ions and K/Na ratio as determined by electron probe X-ray microanalysis (EPXMA) for HOMF incubated in different lidocaine concentration for increasing time periods

		Na		Mg		P		S		Cl		K		Ca		K/Na
Control		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean
	30 min	41.235	13.963	20.246	4.161	210.798	25.830	67.829	16.049	159.016	32.994	332.859	45.709	21.442	6.997	8.072
	1 h	42.275	14.893	25.958	5.619	299.464	41.948	81.793	21.461	165.206	30.121	360.658	58.265	22.162	7.490	8.531
	6 h	37.535	13.745	18.685	5.880	233.956	34.241	70.210	19.733	119.158	19.768	283.643	83.869	9.069	10.942	7.557
	12 h	46.624	13.157	18.667	6.553	307.710	48.381	68.280	16.873	181.449	19.550	412.526	42.414	12.329	8.007	8.848
	24 h	50.761	13.430	20.835	4.369	293.802	22.030	69.369	9.872	184.801	26.905	408.322	52.242	16.763	7.998	8.044
	48 h	42.555	20.262	21.152	11.690	319.404	68.848	76.720	8.187	177.539	33.268	359.307	38.479	10.964	7.743	8.443
DNA TD_{50}	30 min	172.492	81.823	43.807	31.380	231.969	104.204	43.516	35.453	92.145	53.672	83.900	58.406	7.212	22.336	0.486
	1 h	121.392	41.226	17.916	4.083	131.526	48.212	74.074	19.375	226.580	71.698	137.611	38.417	36.295	17.663	1.134
	6 h	92.366	41.281	17.688	8.139	240.489	99.989	62.603	28.198	103.335	58.480	14.656	15.244	50.263	32.546	0.159
	12 h	173.102	91.209	13.347	6.814	232.523	68.367	64.251	22.854	303.150	190.305	17.637	17.786	47.581	50.866	0.102
	24 h	140.826	67.019	38.173	20.935	255.459	97.226	56.934	32.112	83.284	50.854	68.767	89.863	24.518	37.286	0.488
	48 h	189.785	139.801	58.296	44.847	229.340	92.686	78.844	41.402	74.910	140.565	32.649	119.170	23.956	22.022	0.172
WST-1 TD_{50}	30 min	29.458	10.208	15.567	4.622	139.039	21.628	50.128	6.081	95.053	31.560	84.213	31.886	62.650	21.843	2.859
	1 h	51.710	26.109	24.270	12.033	213.558	105.125	80.787	38.943	125.947	64.236	123.644	64.679	47.218	30.618	2.391
	6 h	51.307	33.390	17.174	3.885	215.493	24.264	53.646	18.216	151.683	44.922	216.754	57.767	15.571	5.443	4.225
	12 h	63.578	50.955	24.664	7.758	294.715	43.295	78.865	28.678	242.124	105.350	286.666	81.494	10.526	12.094	4.509
	24 h	63.053	19.552	25.358	5.512	271.343	30.575	48.305	7.045	105.783	26.701	233.552	47.506	19.608	6.290	3.704
	48 h	380.351	482.529	38.328	20.907	121.368	79.082	61.158	28.463	219.207	297.702	11.341	22.223	38.946	74.533	0.030
MTT TD_{50}	30 min	64.460	18.106	19.799	3.838	222.725	22.676	57.303	10.210	160.129	25.236	255.124	34.404	14.756	6.662	3.958
	1 h	63.375	22.579	22.612	7.665	249.545	38.847	73.224	19.600	145.542	33.197	239.887	49.040	16.588	13.110	3.785
	6 h	52.287	32.369	17.112	10.019	216.912	46.294	57.456	19.395	153.426	50.461	158.899	74.391	14.639	20.671	3.039
	12 h	74.802	55.648	27.963	13.476	256.547	53.069	47.742	18.360	136.159	64.217	231.068	142.607	8.226	11.313	3.089
	24 h	86.975	37.257	38.053	16.210	203.101	46.339	37.767	18.198	126.775	73.753	169.810	59.863	4.873	15.330	1.952
	48 h	170.085	80.558	41.480	15.127	248.382	156.278	58.566	38.559	61.345	70.712	27.604	27.897	31.968	102.471	0.162

SD: standard deviation.

is poorly understood. For this reason, one of the screening methods used in the present work was EPXMA, which allows the efficient analysis of cell death mechanisms and processes.[21,42,43] This technique simultaneously analyzes the intracellular concentrations of the main cell ions and the ultrastructure of the cells,[18] and it can be used to identify the cellular effects produced by cell exposure to biomaterials or a foreign substances.[17] Although a previous study has determined the effects of lidocaine on human epidermis by using EPXMA,[44] this method has not been used for the analysis of the local anesthetic on human oral cells *in vitro* or for the evaluation of a wide range of dosages and incubation times.

Some reports have demonstrated that intracellular ion homeostasis plays a pivotal role on cell viability and cell function,[45] with K/Na ratios being very sensitive microanalytical indicators of cell viability and cell metabolism.[17] The use of this method on human oral mucosa cells treated with lidocaine revealed that the highest K/Na ratios corresponded to control cells incubated in DMEM medium without lidocaine. When the cells were incubated in WST-1 and MTT TD_{50} concentrations, the K/Na ratio significantly decreased at most incubation times. Finally, the highest dosage corresponded to DNA TD_{50}, and the EPXMA analysis revealed significantly low ratios at all times. All these results point out that lidocaine is able to alter cell viability and cell functionality in a dosage-dependent manner, and DNA TD_{50} concentrations would result in an important decrease of cell viability as determined by this highly-sensitive method. Again, the effect of culture time is less intense than lidocaine concentration.

In addition, EPXMA associated to scanning electron microscopy demonstrated that culture of HOMF with lidocaine at TD_{50} was not able to induce structural cell damage, although cell function was significantly impaired. This is supported by the adequate maintenance of P intracellular concentrations at TD_{50} concentrations. The content of P is a good indicator of cell membrane integrity[16] and its intracellular variation could be associated to shape changes.[45] In addition, S has been recognized as a marker of sulfurated cellular protein content.[17] Maintenance of the intracellular S contents suggests that the function of these proteins could also be preserved at these concentrations. In the same sense, the analysis of Mg contents suggests that the ATP concentration could also be preserved.[21,22] EPXMA also revealed a decreasing tendency of the Cl ions with culture time, and this finding was compatible with a pre-apoptotic cell state as previously demonstrated by numerous researchers.[16] In agreement with our results, a reduction in Cl and K contents and an increase of Na contents were evidenced in cells showing typical morphological features of apoptosis.[16]

Taken together, our microanalytical analysis revealed that the cell death mechanism that is associated to lidocaine TD_{50} is apoptosis. Our combined approach of cell viability and cell function analysis suggests that caution should be used when applying lidocaine in dentistry, including the intra-articular injection of this anesthetic in the TJM, periodontal ligament space, dental pulp and other areas where wound healing is required, since apoptosis may be activated by lidocaine.

The present study has some limitations. On the one hand, the controls used in the study were based on cells incubated in DMEM culture medium, and commercially-available lidocaine was diluted in the same medium. New studies should be carried out using purified 100% lidocaine diluted in an inert pharmacologically inactive substance or excipient to determine the real role of lidocaine in cell toxicity using more comparable study groups. On the other hand, our *in vitro* results cannot be directly extrapolated to the clinical setting, and *in vivo* studies should be further performed in animal models. Finally, further studies should be carried out to evaluate the cytotoxicity of other anesthetics such as dibucaine, tetracaine, bupivacaine, ethylaminobenzoate, procaine, mepivacain and articaine, prilocaine and ropivacaine to compare them with lidocaine.

In conclusion, the combination of methods and techniques described in this work demonstrated to be efficient for the evaluation and screening of the cytotoxic and metabolic effects of a local anesthetic, and opens the door to the analysis of other anesthetics, drugs and other types of compounds using the same comprehensive methodology. The results of the lidocaine analysis imply that this anesthetic may be capable to exert immediate or delayed damaging effects at clinical concentrations. Therefore, care should be taken when lidocaine is clinically used, and the lowest efficient concentrations should always be used. Furthermore, these results could be valuable in future drug delivery designs.

Author contributions: ACO performed the research and wrote the manuscript. IAR obtained the samples and performed the cell cultures. IG was involved in conception and design and performed some cell viability assays. MAMP assisted in the preparation of the manuscript and in the cell viability analyses. CAAR assisted in the research related to cell culturing and metabolic assays. JMG did the statistical analyses. MCSQ analyzed the cells using EPXMA to determine cell viability. MA was involved in conception and design, data analysis and interpretation and preparation of the manuscript.

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