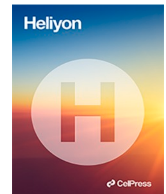
Heliyon 10 (2024) e23060

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Review article

Research methods assessing sodium hypochlorite cytotoxicity: A scoping review

Hernán Coaguila-Llerena, Luana Raphael da Silva, Gisele Faria *

Department of Restorative Dentistry, Araraquara School of Dentistry, São Paulo State University -UNESP, Araraquara, São Paulo, Brazil

A R T I C L E I N F O

Keywords:
Cell culture
Cytotoxicity
Endodontics
Sodium hypochlorite

A B S T R A C T

Sodium hypochlorite (NaOCl) cytotoxicity has been assessed using different methodologies, which has led to arbitrary interpretations. This scoping review aimed to discuss the different methodological protocols for assessing NaOCl cytotoxicity. A literature review performed in the PubMed and Embase databases up to July 2023 identified manuscripts reporting NaOCl cytotoxicity. The dataset identified 546 publications, further screened by 2 reviewers. Ninety studies were identified and mined methodologically to collect information on cell type, cytotoxicity assay, NaOCl dilution solutions, presence of fetal bovine serum (FBS), and NaOCl exposure time. The culture medium used in cytotoxicity assays contains buffering substances that neutralize the pH of NaOCl, thus reducing its cytotoxicity, an approach that may lead to bias when solutions with different pH are compared. For short exposure periods, as in simulations to evaluate the contact between irrigant and periapical tissue cells during chemo-mechanical preparation, NaOCl dilution should be performed with saline, which does not buffer the irrigant. For long exposure periods, as in simulations of irrigant extrusions, NaOCl should be diluted in the culture medium, to reproduce the expected buffering effect occurring in extrusions. The presence of FBS in culture medium can decrease NaOCl toxicity. There is no standardization of NaOCl cytotoxicity methodologies. This poses the risk of arriving at incorrect results and, therefore, pertinent tests must be refined.

1. Introduction

The term “biocompatibility” is widely used in biomaterials science, but its definition has changed over the years [1,2]. Currently, biocompatibility is generally considered the ability of any biomaterial to perform a desired function, without causing undesirable local or systemic effects, but eliciting the most appropriate, beneficial cellular or tissue response, and optimizing the clinical performance, i.e., “biocompatibility = not harmful + efficiency” [3].

According to the traditional paradigm of biocompatibility analysis, dental materials should be subjected to three levels of testing, namely: from the simple to the most complicated method, from *in vitro* to animal testing, and from preclinical to clinical in humans [4,5]. To this end, high-risk materials should be “filtered” early in this process, by using quick and less expensive testing, which can save time, money and possibly animal or human suffering. Some of the materials should also be “prefiltered” by animal testing. Eventually, relatively few materials would reach the clinical testing level [5].

However, early studies using this approach showed that *in vitro*, animal and clinical tests did not work as proposed by the

* Corresponding author. 1680, Zip Code: 14.801-903, Araraquara, SP, Brazil.
E-mail addresses: hernan.coaguila@unesp.br (H. Coaguila-Llerena), lrsodonto@gmail.com (L. Raphael da Silva), gisele.faria@unesp.br (G. Faria).

https://doi.org/10.1016/j.heliyon.2023.e23060
Received 9 March 2023; Received in revised form 26 October 2023; Accepted 24 November 2023
Available online 29 November 2023
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traditional paradigm. There are materials that present *in vitro* toxicity, but perform more favorably in preclinical and clinical tests [5]. That is why the traditional paradigm has undergone changes, and has evolved into a concept proposing that the data obtained from tests at each level should complement one another, and should be evaluated jointly to establish the biological safety of the material [3,5]. In other words, the biocompatibility of a new material should be assessed by employing a battery of standard tests [6], which should include primary (*in vitro*), secondary, preclinical and clinical tests, and should not constitute a single test [3].

Different *in vitro* and *in vivo* tests were described in the scientific literature to evaluate biological reactions to dental materials. Well-known and widely used tests are included in the International Standardization Organization (ISO) 10993 [7,8], and ISO 7405 standards series [3,9,10]. In 1992, the ISO developed the 10993-5 standard, which describes test methods to assess *in vitro* cytotoxicity of medical devices. This is a European regulation subjected to periodic review. Its last version was prepared under a mandate given to the European Committee for Standardization (CEN) by the European Commission and the European Free Trade Association in 2009. This standard was identically adopted by the United States regulations under the supervision of the American National Standards Institute (ANSI) and the Association for the Advancement of Medical Instrumentation (AAMI). In 2014, this ANSI/AAMI/ISO 10993–5:2009 (R2014) standard was revised and ratified with no amendments or corrections [11].

It is essential that researchers use, or at least consult, the relevant standards when evaluating the biocompatibility of any material [3]. Other methodologies not described in these standards, or employing diverse cellular or molecular biology approaches, can also be used [2,3].

In vitro assays allow several phenomena to be studied, including the toxicity of a material at the cellular level, i.e., its cytotoxicity [3,12–17]. Cytotoxicity tests are primary biocompatibility tests that allow evaluating not only cell death or functional changes not leading to death, such as inhibition of proliferation, metabolism, and DNA synthesis, but also other effects caused by certain substances [3,9,18]. This means that cytotoxicity represents but a single aspect of biocompatibility [3]. In general, cytotoxicity tests are used to compare the cellular reactions caused by materials and controls (relative cytotoxicity analysis), and also to determine the possible mechanisms of toxicity of these materials [3,5].

Sodium hypochlorite (NaOCl) is a strong oxidizing, proteolytic, and hydrolyzing agent [19]. Although NaOCl shows high toxicity at high concentrations [17,20], it is considered the gold standard for endodontic irrigation, since it presents suitable organic dissolution and antimicrobial activity [21]. This is why NaOCl is the most commonly used control in cytotoxicity assays when endodontic irrigants (EI) are evaluated.

The effect of NaOCl on cells in culture may range according to the methodological protocol used, implying that the results may be interpreted inappropriately. For example, one study revealed that 2 % CHX was more cytotoxic than 2.5 % NaOCl [22], while another study from the same research group reported the opposite, i.e., 2.5 % NaOCl was more cytotoxic than 2 % CHX [16]. The explanation for this difference in cytotoxicity may be related to the solution for irrigant dilution. While Cassiano et al. [22] used a culture medium that reduced the pH of NaOCl (from 11 to 12 to 7.5–8.0) [15], Coaguila-Llerena et al. [16] used saline solution, which does not modify the pH of NaOCl [15], thereby resulting in higher NaOCl cytotoxicity. Other factors, such as the presence of fetal bovine serum (FBS) in culture medium, cell type, assay type, and NaOCl exposure time can also influence NaOCl cytotoxicity [3,23,24].

Although the ISO standard is used as a reference, methodological variations have been carried out in the investigation of NaOCl cytotoxicity. It can be questioned why researchers do not employ ISO 10993-5 (2009) or AAMI/ISO 10993–5:2009 (R2014) international standards for assessing the NaOCl cytotoxicity. These standards recommend that the cytotoxicity of a medical device be quantitatively assessed by measuring cell death, inhibition of cell growth, cell proliferation or colony formation using, for example, MTT, XTT, neutral red or colony formation assays. Additionally, they describe the exposure time of the cells to the substance, the use or not of FBS, among other issues. However, both standards do not address the specific assessment of NaOCl cytotoxicity, whose composition and usage characteristics may lead to variations in methodologies, and, sometimes, inappropriate interpretation of its cytotoxicity. In this regard, the present scoping review discusses the different methodological protocols for assessing NaOCl cytotoxicity.

Table 1
Assays used for NaOCl cytotoxicity assessment[a].

Type of assay	n	%
MTT	49	55.1
XTT	4	4.5
MTS	10	11.2
WST-1	3	3.4
Sulforhodamine B	4	4.5
Neutral red	8	9.0
Trypan blue	7	7.9
alamarBlue	6	6.7
Tritium-labeled thymidine	2	2.2
Chromium release	3	3.4
CyQUANT	1	1.1
Calcein AM	1	1.1
Bradford method	1	1.1
Lactate dehydrogenase	4	4.5
Live/Dead	5	5.6

[a] More than one assay was used in some studies.

1.1. Bibliographic search

A bibliographic search was performed in the Pubmed and Embase databases, using the following keywords and Boolean operators: ("cytotoxicity" OR "cell culture") AND "sodium hypochlorite", up to July 2023. Initially, 546 studies were identified, after which the abstracts and full texts were critically analyzed by 2 reviewers independently. Eighty-nine studies were identified, and methodologically mined to collect information on cytotoxicity assays, cell types, solutions used for NaOCl dilution, presence of fetal bovine serum (FBS), and NaOCl exposure time.

1.2. Cytotoxicity tests

The text results are shown in Table 1. The methyl-thiazol-tetrazolium (MTT) assay, a tetrazolium salt-based colorimetric test, was the test most commonly used to assess cytotoxicity (55.1 % of the papers) [15–17,23], [25–40], [41–68]. This assay measures cell viability based on the reduction of tetrazolium salt to water-insoluble formazan crystals by dehydrogenases of mitochondria of living cells [69]. Other tetrazolium salt-based tests, such as XTT, MTS or WST-1, have also been used to assess the cytotoxic effects of diverse substances (19.1 % of the papers) [24,70–85]. The neutral red (NR) uptake assay, used in 9.0 % of the papers [16,17,25,27,29,42,67,86], uses a dye to measure cell viability based on how well viable cells can retain it in their lysosomes [87]. The sulforhodamine B (SRB) assay, used in 4.5 % of the papers [88–91], is based on measuring cellular protein content [92]. Other tests such trypan blue, alamarBlue, tritium-labeled thymidine, chromium release, CyQUANT®, Calcein AM, Bradford method, lactate dehydrogenase (LDH), and Live/dead have also been conducted to assess NaOCl cytotoxicity [22,26,31,35,36,50,66,67,86,93–109]. According to the ISO 10993–5:2009 standard, the cytotoxicity screening of medical devices should be performed with the MTT assay, the XTT assay, the NR uptake assay or the colony formation cytotoxicity test; however, other assays could be conducted if a cytotoxic effect is detected [7].

Although all of the assays described above have advantages and specific cellular targets, there is no ideal method, since all of them have limitations. For example, in the case of the MTT assay, high values may indicate that the cells are metabolizing intensely, or "fighting" to survive against a less aggressive material [110]. Additionally, cells primarily in early stages of apoptosis still have partially intact succinate dehydrogenase activity, which can be erroneously considered as cell viability. Some conditions or compounds can also increase or decrease respiratory/mitochondrial activity, resulting in increased or decreased succinate dehydrogenase activity, which can lead to false negative or false positive results [111]. This reinforces the importance of performing more than one assay to evaluate more than one cell parameter. Regarding the SRB assay, it does not distinguish between viable and dead cells [92,112]. In the case of NR uptake, the main limitations regard its lipophilic characteristics, because it may bind to certain test samples or adsorb components such as carbon black [6]. The trypan blue assay does not allow differentiating between living cells and those that are alive but losing function [112]. In the case of alamarBlue, it is possible that there is fluorescent interference from the test compounds, which can be misinterpreted as toxic effects on cells [112]. The main limitation of LDH assay is that FBS and some other compounds have inherent LDH activity [112]. The main drawbacks of the Live/Dead assay are the need for a fluorescent plate reader and the relatively high price of fluorescent dyes [113].

Several studies have used only one assay to assess the cytotoxicity of EI. This is a critical aspect to be considered. More than one assay is a more valid approach to analyze the cytotoxicity of a dental material, because a more wide-ranging assessment using different cellular parameters can reduce the respective disadvantages of single tests, and can complement the results [3,114]. Moreover, a single MTT is no longer accepted by some scientific journals [3].

1.3. Cell types

Cytotoxicity tests are conducted using primary cell cultures [22,24,38,43,44,47,49,54,60,65,67,71–73,75,77–79,81–83,86,100,105,106,108], permanent cell lines [15,17], [23,25–37], [39–41], [45,46,48], [51–53], [55–59], [61–64], [68,70,74,85,88,89], [93–97], [99,101,102,104,107,109,115], or both [16,42,50,66,76,80,84,90,91,98,103]. Most of the papers assessing NaOCl cytotoxicity were conducted with permanent L929 fibroblasts (Table 2). Specific primary cell cultures from human dental pulp [47,54,81,82], apical papilla [22,49,75,90,91,98], osteoblasts [25,76,79,108], periodontal ligament [16,22,38,42,43,50,65,71,72,75,78,80,81,98], gingiva [78,81,83,100,105], or exfoliated deciduous teeth [60] have also been used. Other sources of primary cultures, such as tooth germs, skin fibroblasts, dermal fibroblasts, keratinocytes, epithelial cells, umbilical vein endothelial cells, peripheral blood mononuclear cells, pulmonary fibroblasts, etc., are further alternatives [24,26,44,45,66,67,77,84,86,91,95,103,106,108].

ISO 10993–5:2009 suggests using permanent cell lines [7] to screen a new material by comparing it to a large number of known materials (relative cytotoxicity analysis) [3]. However, primary cell cultures should be used when specific sensitivity must be determined [6,7]. Furthermore, it is important to use cells derived from the site of interest when evaluating specific reactions to a particular material [3]. The main advantage of using primary cultures is that they have high a specificity potential, almost like the

Table 2
Cell types used for NaOCl cytotoxicity assessment.

Type of cell line	n	%
Permanent	52	58.4
Primary	26	29.2
Both	11	12.4

target cells of dental materials in tissues [18]. However, a primary culture is labor-intensive, time-consuming, exhibits low cell profitability, and has a limited passage number [116].

An interesting strategy could involve using different types of cells that may be exposed to EI in a clinical situation, and simulating teeth with open apex, or EI extrusion. The rationale for this strategy lies in the fact that different cells can be affected by various degrees of severity, depending on the target mechanism of cytotoxicity of the material/substance [16,117].

1.4. Solution used for NaOCl dilution

Irrigating solutions may not be applied at the same concentration when used clinically. This is because the cells in culture are more sensitive to the toxicity of a drug than the variety of elements in the periapical tissues, which contain phagocytic cells, and both lymphatic and blood channels that help dilute and transport the drug [118]. ISO 10993–5:2009 standard recommends diluting the solutions in a culture medium [7], as reported by the vast majority of papers (60.7 %) [22,24], [26–28], [30,32–34], [37–41], [43,44, 46–48], [50,53,55–57], [59,60,62,65,66], [70–74], [77,78], [82–84], [86,88–91], [94–102,115], as seen in Table 3. However, the use of a culture medium or even phosphate-buffered saline (PBS), both of which contain buffering substances, may modify the pH of irrigants, e.g., the pH of NaOCl (>11.0) is reduced to 7.5–8.0 [15,119]. These buffering substances are a critical parameter in studies comparing irrigants with different pH values, because EIs do not contain pH neutralizers in a clinical situation. Acidic or alkaline pH values can be directly related to the cytotoxicity of the irrigant [14,120].

A factor that must be borne in mind is the hard-tissue debris formed during chemo-mechanical preparation, and responsible for decreasing the pH of NaOCl. A study showed that dentin powder significantly lowered the pH (from approximately 10 to 8) of 2.5 % NaOCl over a 10-min evaluation period [121]. Another study revealed that the pH of 3 % NaOCl dropped from 12 to <10 in the presence of dentin [122]. However, it is important to note that irrigants are constantly being replenished during a treatment session that uses conventional syringe irrigation or any other agitation method, thus renewing the original NaOCl, which has higher pH [123, 124]. Nonetheless, EI dilutions in buffered solutions could lead to non-reliable results. Some studies have been conducted with saline (5.6 % of the papers) [15–17,29,42], which does not contain buffering substances, in order to offset the culture medium/PBS disadvantage (Table 3). For example, NaOCl and CHX at similar concentrations have comparatively divergent cytotoxic effects, according to whether they are diluted in saline or culture medium [17,22].

It is important to note that if NaOCl is diluted in saline solution, there should be short cell incubation periods, e.g., 10 min, since saline solution does not contain nutrients [15–17,33]. This does not represent a problem when simulating root canal preparation, since the process ranges from 3 to 15 min [42,82]. A total of 20.2 % of the papers did not specify the solution in which the NaOCl was diluted. This is a gap that needs to be specified in future studies.

Another aspect to be considered is the effect of fetal bovine serum (FBS) on the cytotoxicity of certain materials. Although the cytotoxicity of dental materials is assessed in the presence of FBS [22–24,27,28,32–34,41,50,57,59,60,65,71,73,77,83,86,89,94,95, 99,100,115], as observed in 25.8 % of the papers (Table 3), this reagent could alter the results. It has been shown that the presence of FBS in the culture medium decreases the cytotoxicity of chlorhexidine, hydrogen peroxide and NaOCl. This effect is probably related to the protective effect of FBS itself, or the reaction of dental materials with FBS components, and triggers an increase in the organic matter [23,24]. Another study showed that the NaOCl dilution medium did not induce any cytotoxic effects on L929 fibroblasts, whether or not FBS was used. The authors explained that using FBS could simulate *in vivo* tissue damage of periapical tissues (irrigant extrusions) [27]. Since FBS does not necessarily have to be used according to the ISO standard, its variable use in studies yields contrasting results, hence calling for more research. Unfortunately, most papers (39.3 %) do not clearly specify whether or not FBS was used (Table 3), and this makes it difficult to analyze this issue appropriately.

Table 3
NaOCl dilution and presence of fetal bovine serum (FBS) for cytotoxicity assessment.

NaOCl dilution	n	%
Culture medium	54	60.7
Saline	5	5.6
PBS	5	5.6
DPBS	1	1.1
Distilled water	2	2.2
No dilution	1	1.1
DMSO	1	1.1
Sodium bicarbonate solution	1	1.1
Not specified	18	20.2
FBS presence	**n**	**%**
Yes	23	25.8
No	30	33.7
Both	2	2.2
Not specified	35	39.3

1.5. NaOCl exposure time

Traditionally, diluted irrigating solutions used in cytotoxicity assays remain in contact with the cells for 24 h [12,13] as recommended by ISO 10993–5:2009 [7]. This was observed in almost the half of the papers (46.2 %) [24,26–28,32,35–37,40,43,45,47,48,50,55,56,59,61,62,65,66,70,73,74,76–78,81,83,88–91,97,98,102,109] (Table 4). However, in the case of NaOCl, studies have been conducted using less than 24 h contact periods [15–17,23,25,29–31,33,34,41,42,49,51,52,57,58,60,63,68,72,79,82,84,94,99–101,105–108]. The use of prolonged exposure periods of NaOCl is questionable; one study reported that NaOCl caused decreased cell viability in 8 h of contact with fibroblasts, and went on to assert that this effect may be reversible, since these fibroblasts recovered after being exposed to NaOCl for 24 h [24]. This recovery can be attributed to the rapid decomposition kinetics of chlorinated solutions such as NaOCl; i.e., the maximum release of chlorine in the cell culture occurs in the first 4 h, after which the actual concentration of hypochlorous acid (a strong oxidant causing cell death) decreases progressively and inversely proportional to the exposure time [24]. However, long exposure periods could reproduce a clinical situation of EI extrusion. To this end, NaOCl must be diluted in the culture medium in order to reproduce the expected long-term buffering occurring in extrusions [22]. On the other hand, a short exposure time, such as 3–10 min, can simulate the contact between the EI and cells occurring during chemo-mechanical preparation (i.e., periodontal ligament, pulp, apical papilla). In these cases, NaOCl should be diluted in saline, which does not buffer the EI [17].

In some studies, the EI remained in contact with the cells for the entire evaluation period [24,41,76], while, in others, the EI was removed after short contact periods, and the cells were incubated with culture medium for periods of 4 h [15–17,33] or 24 h [82]. The 4-h incubation period of cells in culture medium is the ideal period for studying cell death in cell culture, since apoptotic cells are not phagocytosed in a culture incubated for longer periods, which risk causing cell necrosis secondary to apoptosis [111,125]. Based on this rationale, the following question may be posed: Why not perform the cytotoxicity assessment immediately after removal of the EI? The answer is that a short period of roughly 4 h [15–17,29] is needed for cellular phenomena to become identifiable.

1.6. Clinical extrapolation

The direct extrapolation of cytotoxicity test results to the clinical situation is usually not possible, even when wishing to simulate an *in vivo* situation [3]. In *in vitro* tests, the fluid flow is static, unlike the dynamic and homeostatic *in vivo* situation [6]. However, cytotoxicity tests are required to perform the initial analysis of new materials, particularly to compare the cellular reactions of the new materials to those of known materials (relative cytotoxicity analysis), and to guide the sequential performance of other tests [3,6,18]. Technical advances have allowed 3D tissue-like cultures and co-cultures, which can contribute to assessing cytotoxicity and material interaction pathways, to be performed in a promising, predictive manner [126,127], by providing the metabolic and morphological evaluation of cells and their interactions, a procedure similar to evaluating *in vivo* conditions [9]. However, despite the improvement in *in vitro* tests, animal tests are still needed to evaluate the biological response to dental materials.

2. Conclusion

There is no standardization of NaOCl cytotoxicity methodologies. This poses the risk of arriving at incorrect results. Therefore, pertinent tests must be refined to offset test limitations.

Data availability statement

Data will be made available on request.

Table 4
NaOCl exposure time for cytotoxicity assessment.

Type of assay	n	%
Up to 14 days	1	1.1
Up to 7 days	2	2.2
Up to 5 days	1	1.1
Up to 72 h	3	3.4
Up to 48 h	9	10.1
Up to 24 h	41	46.1
Up to 18 h	1	1.1
Up to 12 h	1	1.1
Up to 4 h	1	1.1
Up to 3 h	2	2.2
Up to 1 h	4	4.5
Up to 30 min	2	2.2
Up to 15 min	8	9.0
Up to 10 min	9	10.1
Up to 3 min	2	2.2
Up to 2 min	1	1.1
Not specified	2	2.2

CRediT authorship contribution statement

Hernán Coaguila-Llerena: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Luana Raphael da Silva:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Gisele Faria:** Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by fellowships from Dean of Research (PROPe)/Dean of Postgraduate Studies (PROPG) of Sao Paulo State University (UNESP) – Call notices 04/2022 and 01/2023, and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) - Grants #2018/24662-6 and #2022/07823-1.

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