

# Effect of nicotine on human gingival, periodontal ligament and oral epithelial cells. A systematic review of the literature

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## ABSTRACT

**Objectives:** A systematic review was conducted to evaluate the *in vitro* effects of nicotine on human gingival, periodontal ligament and oral epithelial cells, specifically: cell viability, cell attachment, cell proliferation and inflammatory mediator production.

**Materials and methods:** This report followed the PRISMA statement. Two reviewers performed a literature search up to October 2018 in 3 databases: MEDLINE, EMBASE and Web of Science, supplemented by manual searching. Inclusion criteria comprised: *in vitro* studies evaluating human gingival fibroblasts, human periodontal ligament cells or human gingival/oral epithelial cells; nicotine exposure as a variable; including an appropriate control (no nicotine); published in English. Quality assessment was based on a 15-item checklist.

**Results:** Of 356 potentially eligible studies, 42 were included. The median quality assessment score was 8/15. Study designs were highly heterogeneous. IC<sub>50</sub> values for nicotine (exposure concentration causing 50% cell death or inhibition of cell growth or other utilised toxicity metric) derived from ten studies ranged from 6 μM to 25 mM. Studies investigating cell attachment, proliferation and inflammatory mediator production suggested that effects can be seen at a wide range of nicotine concentrations, but results were often contradictory.

**Conclusions:** According to findings from *in vitro* studies, nicotine, at levels found in tobacco smokers, nicotine replacement therapy users and e-cigarette users, is unlikely to be cytotoxic to human gingival and periodontal cells, though saliva levels in smokeless tobacco users may be high enough to achieve cytotoxicity. There was limited and contradictory evidence for nicotine effects on cell attachment, proliferation and inflammatory mediator production.

**Clinical significance:** It is well established that whole tobacco smoke is highly damaging to oral tissues. The specific effects of nicotine are not well understood but are of increasing importance given the recent popularity of novel nicotine products. Increased knowledge on this topic will help to better inform dental professionals and patients.

## 1. Introduction

Smoking is highly prevalent in many populations worldwide. The harmful effects of smoking on general health have been well documented [1]. Smoking also has significant adverse effects on oral health, with a broad literature documenting the relationship of smoking to periodontal disease [1–4], wound healing and oral cancers [1].

Smoking is a major risk factor for periodontitis. Smokers are more likely to suffer from periodontitis (OR: 2.14, 95% CI: 1.44–3.17) and tooth loss (OR: 1.5, 95% CI: 1.25–1.81) than non-smokers [1]. Smokers have poorer responses to both non-surgical and surgical periodontal

therapies compared to non-smokers [5–7]. Similarly, it has been shown that smokers who quit smoking during periodontal therapy achieve improved clinical outcomes compared with those who continue to smoke [8,9].

The literature relating to the *in vitro* effects of tobacco smoke on oral cells and tissues has been the subject of two previous review articles [10,11]. Palmer et al. [11] reviewed the potential biological mechanisms underlying the effects of tobacco smoking on periodontitis. With regards to nicotine specifically, they concluded that ‘nicotine may be unfairly blamed’ for most of the noxious and toxic properties of tobacco smoking. In previous decades, this may have been of little consequence,

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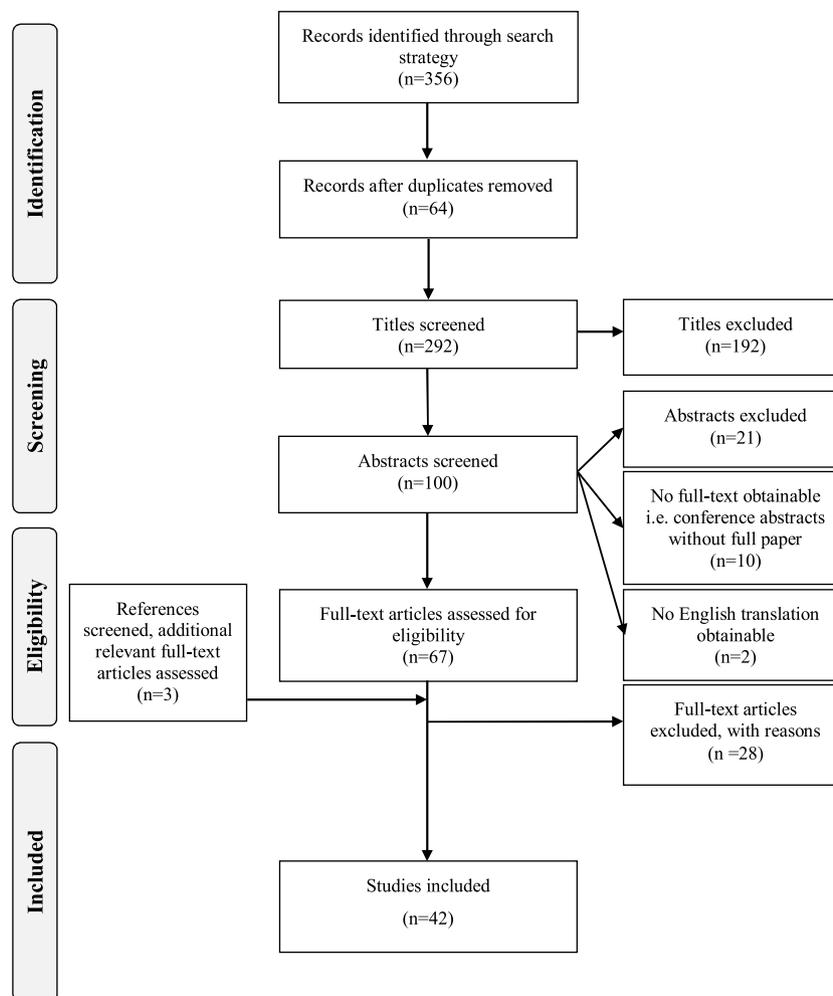


Fig. 1. Flow chart of included and excluded studies (PRISMA).

as labelling nicotine as harmful was useful in presenting tobacco control public health messages. However, with the more recent availability of nicotine replacement therapy (NRT) and new nicotine delivery technologies (such as e-cigarettes), the specific effects of nicotine on oral tissues and cells is of clear relevance and a critical research question.

To the best of our knowledge there has not been a systematic review conducted on the *in vitro* effects of nicotine on oral and periodontal cells despite the large number of studies published. The aim of this systematic review, therefore, was to evaluate the *in vitro* effects of nicotine on human gingival, periodontal ligament and oral epithelial cells, specifically: cell viability, cell attachment, cell proliferation and inflammatory mediator production.

## 2. Materials and methods

The PRISMA statement, checklist (Supplementary Fig. 1) and flow diagram (Fig. 1) were utilised in this review. A search protocol was developed *a priori* following discussion between all members of the research team. The focussed question for the systematic review was: In *in vitro* conditions, does nicotine exposure, compared to no nicotine exposure, lead to changes in cell viability, attachment, proliferation or inflammatory mediator production in human gingival and periodontal fibroblasts and epithelial cells? This question was constructed according to the PICOS framework (Table 1). There is no registration system for non-clinical systematic reviews. The review protocol can be obtained by emailing the lead author.

Table 1

Focused research question presented using the PICOS framework.

PICOS	
Population/patient	Human gingival and periodontal fibroblasts and epithelial cells
Intervention/indicator	Nicotine exposure
Comparator/control	No nicotine exposure
Outcomes	Cell viability Cell attachment/adhesion Cell proliferation Inflammatory mediator production
Study design	<i>In vitro</i> , experimental

### 2.1. Criteria for considering studies for review

Inclusion criteria for studies were: (i) primary studies on human gingival fibroblasts (HGFs) or human periodontal ligament cells (HPDLCs) or human gingival/oral epithelial cells (HGECs/HOECs); (ii) nicotine exposure as a variable; (iii) inclusion of an appropriate control (no nicotine); and (iv) published in the English language. Animal studies and abstracts without full papers were excluded.

### 2.2. Search methods for identification of studies

#### 2.2.1. Electronic searching

The search terms employed in this study were: ((perio\$ [All fields] OR gingiva\$ [All fields]) AND (fibroblast\$ [All fields] OR epithelia\$

[All Fields]) AND nicotine). MEDLINE, EMBASE and Web of Science were searched without language restriction up to, and including 31<sup>st</sup> October 2018.

### 2.2.2. Unpublished data and hand-searching

Unpublished data were sought by searching a database listing unpublished studies ([www.opengray.eu](http://www.opengray.eu)). Additionally, reference lists of any potential studies were examined (*i.e.* hand searching) in an attempt to identify any further studies that could be considered for inclusion. Bibliographies of review articles, relevant texts, World and European Periodontology Workshops were also screened. A manual search was performed of the Journal of Clinical Periodontology (1974–2018), Journal of Periodontology (1944–2018) and Journal of Periodontal Research (1966–2018).

### 2.3. Data collection, extraction and management

Titles and abstracts from the electronic searches were imported into EndNote X8 (Thomson Reuters, New York City, NY, US). Duplicates were eliminated. Titles were screened independently by two reviewers (RH and JC) and those indicating no relevance to this study were excluded. Abstracts were then screened independently by two reviewers (RH and JC). The full texts of potentially eligible studies were then reviewed against the inclusion/exclusion criteria independently by the two reviewers and disagreement resolved by discussion and consultation with the third author (PMP) when necessary. Data were extracted independently from the full text articles by two reviewers using a piloted data extraction form. Data collected comprised: year of publication, location of first author, funding source(s), cell types studied, 15-item Consolidated Standards of Reporting Trials (CONSORT) quality assessment, cell viability data (assay used, nicotine exposure conditions and results), cell attachment data (assay used, nicotine exposure conditions and results), cell proliferation data (assay used, nicotine exposure conditions and results) and inflammatory mediator production data (assay used, nicotine exposure conditions and results).

### 2.4. Assessment of quality in included studies

A quality assessment was completed on each included study. A 15-item modified CONSORT checklist was used [12]. Developed by Faggion [12], this checklist is designed for assessing the quality of *in vitro* pre-clinical research and gives a score out of 15 (with a higher score indicating higher quality).

### 2.5. Data synthesis

Data were collated into evidence tables, with study characteristics, details of the exposure conditions, details of the assays conducted, and quality assessment included. For data analysis, a narrative approach was utilised.

## 3. Results

### 3.1. Search results and characteristics of included studies

The flow chart of manuscripts screened is shown in Fig. 1. A total of 356 potentially eligible studies were identified by the search strategies. Following removal of duplicates, title screening was completed on 292 studies with 192 studies being excluded at this stage. Abstract screening was completed on 100 studies with 67 progressing to full-text review. An additional three studies were identified from the references of the screened studies. Finally, 42 studies were included in the full data analysis. Supplementary Table 1 provides reasons for exclusion of reviewed full-text studies.

The characteristics of the included studies are presented in Supplementary Table 2. They were published over a 22 year period

(1995–2017). Studies were conducted in 13 different countries (based on first author location) with researchers in the USA [13–25], South Korea [26–32] and Japan [33–37] publishing the greatest numbers of studies per country.

### 3.2. Quality assessment

The median (interquartile range [IQR]) quality assessment score of the 42 assessed papers was 8.0 [1.0], out of a maximum possible score of 15. The lowest score was six [38,39] and the highest score was nine [14,16,19,20,22,32–37,40–45]. Regarding the key methodological domains assessed by the modified CONSORT checklist, all of the studies failed to achieve: sample size determination, random sequence generation, allocation concealment, implementation details, blinding and publication of the full study protocol. Supplementary Table 3 provides full details of the quality assessment for each study.

Regarding funding sources for published studies, seven studies did not provide any details about funding [13,18,24,39,46–48], two stated that they had received no funding [26,41], one detailed an individual providing equipment [38], one was funded by a tobacco manufacturer [15], one was funded by a tobacco endowment fund [14], and the remainder received funding from various educational, governmental and charitable sources (Supplementary Table 2).

### 3.3. Cell types investigated

The most studied cell type were HGFs, being investigated in 24 studies [13,14,19–26,32–34,36,39–41,43,46–51]. Sixteen studies investigated HPDLCs [18–20,25,27,30,31,33,34,38,39,44,45,52–54]. HGECs were investigated by eight studies [15–17,28,29,35,37,42], with one study using HOECs [50]. Supplementary Table 2 provides details of the origin of the cells.

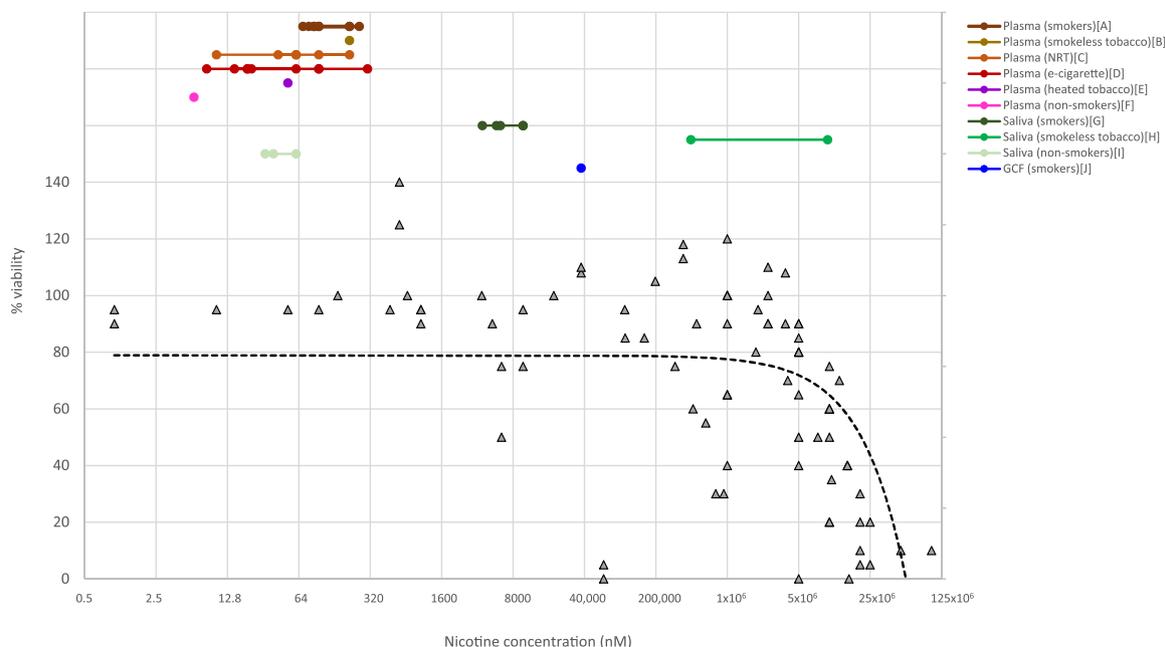
### 3.4. Nicotine exposure

Exposure conditions varied among the studies with respect to nicotine concentrations used and the duration of exposure. The nicotine concentrations varied from 1 nM [23,41] to 100 mM [48], with a mean (SD) of 4.8 mM (10.9) (median and mode: 1 mM). The exposure time varied from 30 min [20] to 4 weeks [21] with a mean (SD) of 44.0 h (54.2) (median and mode: 24 h). Typically, a single dose was administered as diluted pure nicotine added to culture medium. Four studies reported subsequent repeat doses of nicotine, all at 24 h intervals [13,39,40,46] but only one of these ran a single-dose control [39].

### 3.5. Cell viability

Thirty-three studies investigated cell viability using a range of assays [13,15–17,19,20,22–33,35–37,39–43,46–50,53,55]. Fifteen studies used the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [24,26–32,35,41,42,48,50,52,53], five studies used the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay [16,17,19,20,43], five studies used the trypan blue assay [23,33,39,42,49], two studies used the neutral red assay [40,46], two studies used microscopic observation [13,22], two studies used fluorescent dye assessment [25,39], one study used the sulforhodamine B assay [15], one study used the lactate dehydrogenase leakage assay [47], and three studies investigated cell viability in preliminary experiments (but methodological details were not provided) [22,36,37]. Supplementary Table 4 details the principal results.

There was significant heterogeneity between studies with respect to nicotine exposure conditions. However, 19 studies investigated the effect of a 24-h exposure of nicotine on HGFs [22,23,25,26,32,33,39–41,43,46,48–50] or HPDLCs [25,27,30,31,33,39,52,53] (studies explicitly on cells from smokers were excluded in this analysis). Data were derived from these studies and all data points combined in



**Fig. 2.** Results of cell viability assays carried out on fibroblast cultures (not reported to be from smokers) after 24 h of nicotine exposure; expressed as percentage (of viable cells relative to nicotine-free control) on a logarithmic scale of nicotine concentration. Triangles represent data points derived from the studies; dotted line represents line of best-fit ( $Y = -1.4102 \times 10^{-6} X + 78.926$ ). Normal reported plasma, saliva and gingival crevicular fluid nicotine concentrations are shown for comparison with each marker identifying an analysis from a study. The corresponding references are: A = [57–65]; B = [57]; C = [62–64,70]; D = [59,62,65,71–74]; E = [76]; F = [58]; G = [58,66–68]; H = [69]; I = [58,66]; J = [68].

**Fig. 2.**

### 3.5.1. $IC_{50}$

$IC_{50}$  (defined as the exposure concentration at which there is 50% cell death or inhibition of cell growth or other utilised toxicity metric) values were identified from 18 studies reporting toxicity data over a range of concentrations for HGFs or HPDLCs [23,25–27,30–33,39–41,43,46,48–50,52,53] (studies explicitly on cells from smokers were excluded in this analysis). One study explicitly reported an  $ED_{50}$  value [39], defining this as ‘effective dose<sub>50</sub>, concentration of a substance which damages 50% of cells irreversibly’. A further nine studies [26,30,33,41,48–50,52,53] investigated a sufficient exposure concentration range to allow an  $IC_{50}$  value to be derived from the data (Fig. 3). Eight studies [23,25,27,31,32,40,43,46] did not investigate a sufficient exposure concentration range to allow  $IC_{50}$  determination (Fig. 3). The  $IC_{50}$  ranged from 6  $\mu$ M to 25 mM with a mean (SD) of 9.3 mM (8.4 mM). Two studies reported values in this range for HGFs; Lee et al. [28] reported an  $IC_{50}$  value of 300  $\mu$ M (24 h) while Gao, Prasad and Zacharias [15] reported that an  $EC_{50}$  (‘effective concentration for 50% cytotoxicity’) was not reached with 2.8 mM (24 h).

### 3.6. Cell attachment/adhesion

Five studies investigated cell attachment/adhesion using a range of assays [21,38,41,51,54]. Three studied HGFs [21,41,51] and two studied HPDLCs [38,54] (Supplementary Table 4). Four different attachment surfaces were used in the studies: plastic (culture plates) [38,54], root surfaces (from extracted teeth) [21,41], titanium [51] and glass [21]. Three studies used similar nicotine concentrations (620–920 nM) and reported similar reductions in attachment (approximately 50–60%) although they had very different exposure durations: 6 h [38], 24 h [51] and 4 weeks [21].

Esfahrood et al. [41] reported reduced attachment at a wide range of nicotine concentrations (1 nM, 1  $\mu$ M, 1 mM, 5 mM at 24 h) while James et al. [54] reported an approximately 60% reduction in attachment using high nicotine concentrations (31 mM for 24 h). Both of these

studies [41,54] used a viability assay to quantify cells remaining attached to a plate under various nicotine exposures, and it is therefore not readily apparent whether viability, proliferation or attachment was being measured.

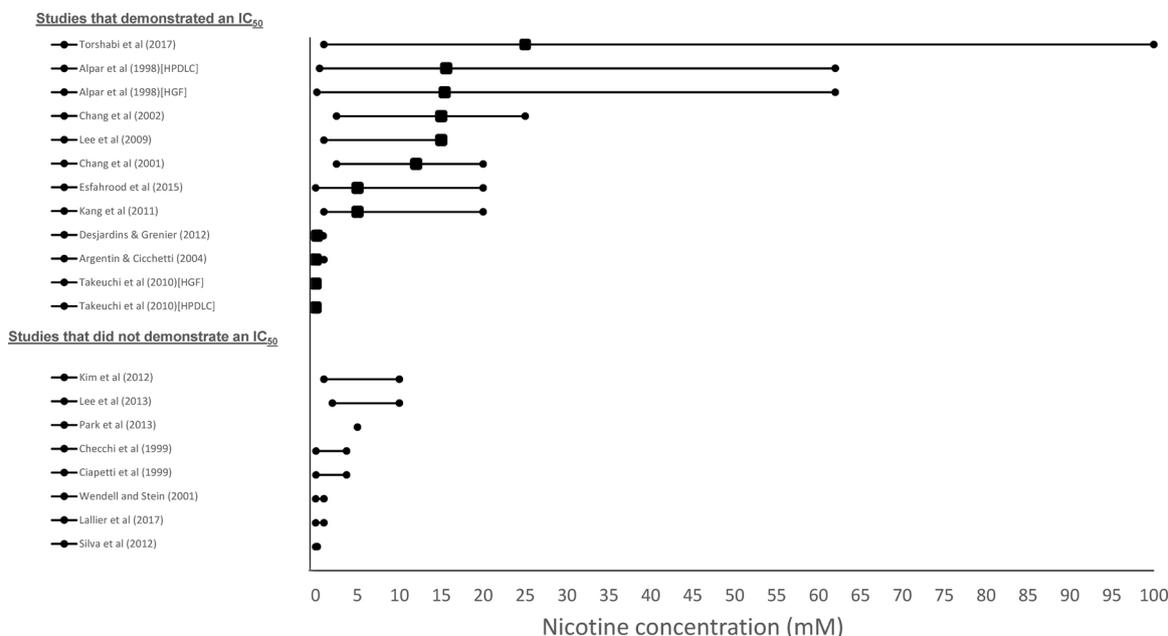
### 3.7. Cell proliferation

Eleven studies investigated the effect of nicotine concentration on cell proliferation. Six studies investigated nicotine effects on proliferation of HGFs [20,22,39,40,46,49], five on HPDLCs [18,20,38,39,53], and two on HGFs [16,28] (Supplementary Table 4).

Nine studies reported reductions in cell proliferation [18,20,22,28,38–40,46,53], one of which also observed increased cell proliferation at a lower nicotine concentration (2.3 mM, 24 h) [18]. Two studies reported inhibition of proliferation at 3.7 mM over 24 h [40] and 48 h [46], respectively, but no statistically significant inhibition at lower concentrations of 37  $\mu$ M and 370  $\mu$ M. Ciapetti et al. [46] observed inhibition only after 48 h exposure, and not at 24 h. Checchi et al. [40] observed inhibition with 3.7 mM over 24 h; this did not apply to the cells sourced from smoking subjects over 40 years of age. Two studies reported no statistically significant effects of nicotine exposure on cell proliferation, at respective exposures of 100 nM, 10  $\mu$ M and 1 mM over 4 and 48 h [16] and 1  $\mu$ M over 24 to 72 h [49].

Of five studies [18,20,38,39,53] which investigated the effect of nicotine on HPDLC proliferation, four studies noted inhibitory effects [18,38,39,53], with one study [20] reporting that proliferation was not significantly affected (6 or 8 mM; 30 min). Chang et al. [53] observed dose-dependent inhibition between 25  $\mu$ M and 200  $\mu$ M after 96 h of exposure to nicotine; all proliferation ceased at concentrations above 400  $\mu$ M. Alpar et al. [39] also found dose-dependent inhibition at concentrations above 3.9 mM after 24 h (all proliferation ceased above 31 mM). Only one study reported an increase in cell proliferation [18] at 2.3 mM after 24 h, with inhibition of cell proliferation observed at the higher nicotine concentration of 9.2 mM.

Four studies also investigated cell proliferation indirectly using wound repopulation, or rate of artificial wound closure [13,14,25,48].



**Fig. 3.** IC<sub>50</sub> of fibroblasts (not reported to be from smokers) after 24 h of nicotine exposure (nicotine concentrations at which a 24 h exposure causes fibroblast viability to diminish to below 50% relative to nicotine-free control); expressed in millimolar (mM) on a linear scale. Squares represent IC<sub>50</sub> values and the line/circles represents the range of nicotine concentrations used in each study.

Three studies used HGFs on culture plates, and one used HPDLCs (Supplementary Table 4). Dino et al. [13] reported a significant reduction in wound repopulation after 4 days of exposure to 4 mM nicotine, and at 6 days with as little as 1 mM nicotine. Lallier et al. [25] reported no effect with up to 1 mM over 5 days.

### 3.8. Inflammatory mediator production

Fifteen studies investigated the effect of nicotine on production of cytokines and other inflammatory mediators relevant in the pathogenesis of periodontitis. Seven studies investigated the effects on HGFs [22–24,34,36,50,51], five on HPDLCs [18,27,34,44,45], four on HGECs [16,17,37,42], and one on HOECs [50]. The included studies investigated between six [24] and one [18,22,36,37,42,45] inflammatory mediator(s). The most common mediator investigated was interleukin-8 (IL-8) [16,23,37,42,44,50,51] followed by interleukin-6 (IL-6) [18,23,50,51], interleukin-1 $\beta$  (IL-1 $\beta$ ) [16,44,45], prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) [16,27,36] and interleukin-1 $\alpha$  (IL-1 $\alpha$ ) [16,17] (Supplementary Table 5).

With respect to IL-8, two studies [17,50] showed no effect on IL-8 production in HOECs, HGECs and HGFs cultures with exposure to a relatively wide range of nicotine concentrations (100 nM - 1 mM; 24 h). Conversely, five studies [23,37,42,44,51] showed increased IL-8 production in HGFs, HPDLCs and HGECs and ‘stimulated HGECs’ cultures with exposure to similar nicotine concentrations (1 nM - 1 mM; 24 h).

Almasri et al. [56] investigated the expression of 23 inflammatory mediators on the HGF cell membrane (rather than production/secretion) and reported that nicotine exposure (1.5 mM, 48 h) resulted in the greatest increased expression of growth-regulating oncogene- $\alpha$  (GRO- $\alpha$ ), interleukin-7 (IL-7) and interleukin-15 (IL-15).

Several studies investigated matrix metalloproteinase (MMP) activity following exposure to nicotine. Tipton and Dabbous [22] reported that collagenase activity in HGFs was statistically significantly increased by nicotine concentrations  $\geq$  1.5 mM with a 6-day exposure. Both Zhou et al. [24] and Takeuchi-Igarashi et al. [34] reported that nicotine exposures (both nM and mM ranges) had no statistically significant effect on MMP-1 and MMP-2 at time frames of up to 48 h. Kim et al. [27] demonstrated an increase in MMP-2 and MMP-9 (1, 5, 10 mM nicotine for 24 h) but no statistical tests were performed.

Takeuchi-Igarashi et al. [34] reported significantly increased tissue inhibitors of metalloproteinases-1 (TIMP-1) levels (in cell culture supernatants) when cells were exposed to nicotine (6.2 nM for 12, 24, and 48 h) whereas Zhou et al. [24] reported reduced levels of TIMP-1 in nicotine-treated cells (1.5 mM, 48 h, no statistical tests performed).

### 4. Discussion

This systematic review identified a large number of studies that have investigated the *in vitro* effects of nicotine on human gingival, periodontal ligament and oral epithelial cells. We observed high heterogeneity between studies particularly regarding the assays performed, the cells studied and the nicotine exposure conditions that were applied, the latter being particularly varied, with nicotine concentrations ranging from 1 nM [23,41] to 100 mM [48] and exposure time varying from 30 min [20] to 4 weeks [21].

The plasma nicotine concentration of tobacco smokers is well established in the literature with reported concentrations usually being in the range of 70 nM to 200 nM [57–65]. Salivary nicotine concentrations of tobacco smokers have been reported to range from 4  $\mu$ M to 10  $\mu$ M [58,66–68] with much higher levels being reported in smokeless tobacco users (0.43–9.62 mM) [69]. Gingival crevicular fluid (GCF) was found to have a nicotine concentration of 37  $\mu$ M in an analysis of seven smokers [68]. Plasma nicotine concentrations in those using NRT have been shown to be similar to, or lower than, those found in cigarette smokers [62–64,70]. With regards to e-cigarettes, several studies reported little effect on plasma nicotine levels [59,62,71,72], most likely because they used early products and novice users. More recent studies have reported plasma nicotine concentrations that are similar to those observed in tobacco smokers: 100 nM [65,73] and 300 nM [74]. We are not aware of any published data on salivary nicotine concentrations in e-cigarette users, although salivary cotinine levels (a metabolite of nicotine) in e-cigarette users have been reported to be similar to those of tobacco smokers [75]. A heated tobacco product (heat-not-burn) has been reported to deliver a plasma nicotine concentration of 50 nM [76]. Non-smokers have been reported to have very low salivary nicotine concentrations (30 nM) [58].

It is noteworthy that many of the studies used nicotine concentrations in the mM range and these concentrations are only observed *in*

*vivo* in the saliva of smokeless tobacco users [69].

The challenge with any *in vitro* research is to interpret the findings in an appropriate way, considering the clinical relevance. For example, when considering cell viability experiments, with nicotine concentrations in the regions observed in smokers, NRT users and e-cigarette users, no effect on cell viability was observed. This suggests that nicotine is not the cytotoxic component of tobacco smoke. Consistent with the findings of an earlier review [10], the identified studies in our systematic review only reported a substantial effect on cell viability at nicotine concentrations above approximately 5 mM, and such concentrations would only be seen *in vivo* in the saliva of smokeless tobacco users. From the identified studies, we derived the IC<sub>50</sub> of nicotine, in HGFs and HPDLs, and with 24-h exposure, to range from 6 μM to 15.6 mM (mean [SD]: 9.3 mM [8.4]). The results of our systematic review therefore indicate that the high salivary nicotine concentrations reported in smokeless tobacco users can be cytotoxic to gingival and periodontal ligament cells *in vitro*, as identified in previous reviews (although periodontal ligament cells are not usually directly exposed to saliva *in vivo*).

It should be remembered that pathological processes are more complicated than simple cell viability assays and in this review we also looked at studies that reported on cell attachment, cell proliferation and inflammatory mediator production. We found that studies reported inhibition of cell attachment with exposure to nM concentrations of nicotine whereas cell proliferation seemed only to be inhibited by higher concentrations of nicotine (in the μM and mM range). Production of inflammatory mediators, including cytokines, appeared to be stimulated by exposure to nicotine at a wide range of concentrations (in the nM to mM range). However, the identified studies often reported contradictory results, which make it hard to draw any definitive conclusions. For example, when considering cell proliferation, for an exposure range between 1–5 mM (24 h), some studies reported inhibitory effects [22,39,40], whilst others reported no effects [16,46] or even increased proliferation [18].

It has been postulated by Checchi et al. [40] that some of the differences between studies may be accounted for by varied culture or nicotine exposure conditions. They also proposed that contradictory findings could result from differences in the cell types studied (for example, whether cells were obtained from smokers or non-smokers, or from younger or older individuals, with or without periodontitis, or were commercially-available cell lines, and whether the cells were exposed to nicotine after a variable number of passages). It has also been suggested that nicotine may have a synergistic action with other substances, for example bacterial toxins [77].

Our quality assessment demonstrated several common deficiencies among the included studies. All lacked any randomisation, allocation concealment and blinding, meaning that there was a high risk of potential bias. This finding is likely to be true for *in vitro* studies more generally which rarely utilise any of these techniques [78,79]. When considering human studies, the randomised controlled trial is considered to have the most robust study design for answering research questions. Similar designs can be utilised for *in vitro* studies to reduce the risk of bias [12,80]. We also noted that many studies failed to mention any limitations of their work. Finally, funding details were absent from a number of the studies, which is particularly important given this field of research potentially involving funding from the tobacco industry. Only one study openly reported being funded directly by the tobacco industry [15], with another funded by a tobacco endowment fund [14].

There are some limitations of our systematic review. We limited the scope to nicotine and did not include cotinine. *In vivo*, nicotine is metabolised to cotinine which has a longer half-life than that of nicotine (11–24 h compared to 30–150 min) meaning that *in vivo*, cells could be exposed to cotinine for longer periods with potentially more detrimental effects. Including cotinine was beyond the scope of this review but would be an important consideration for future study. We did not

attempt to analyse the effect of study heterogeneity (e.g. by comparing cells from smokers/non-smokers or from patients with/without periodontitis), though the scope for doing this was limited given the variable design of the studies that were identified. We limited our systematic review to concentrating on four domains (cell viability, attachment, proliferation and inflammatory mediator production) and there may be other domains that are important, e.g. cell/bacterial interactions. We also included additional information on wound re-population and cytokine expression. Although these were not part of our original inclusion criteria, after evaluating the identified studies we felt they offered additional important information and included the data in the relevant sections. We were unable to establish in which countries the studies were conducted as this was rarely reported. Instead we reported on first author location, accepting this might not always be where the study was performed. There is also potential for publication bias e.g. studies which demonstrated no effect of nicotine on the outcome of interest may have been less likely to be published.

In order to improve the quality and value of research in this field and based on the findings of this systematic review, we make the following five recommendations for future studies:

- 1 Use realistic exposure conditions e.g. nicotine concentrations should ideally be within normal physiological ranges or provide justification for why other concentrations are used. An example of a study that does this well from the current review is Wendell and Stein [23].
- 2 Consider using more realistic model systems (e.g. 3D tissue models) rather than the classic cell culture monolayers (where appropriate and practical) [81].
- 3 Report and consider the influence of cell type and origin e.g. cells from smokers, non-smokers or unknown. An example of a study that does this well from the current review is Checchi et al [40].
- 4 Ensure robust study designs, utilising and reporting sample size calculations, randomisation, allocation concealment and blinding. Mutahar et al [82] is an example of an *in vitro* study that has utilised some, but not all, of these design features.
- 5 Utilise an appropriate reporting guideline or checklist [12,80] and ensure study limitations are discussed and acknowledged and study funding reported.

## 5. Conclusions

From the studies identified in this review, it appears that nicotine at concentrations found in the plasma, saliva and GCF of tobacco smokers, NRT users and e-cigarette users is unlikely to be cytotoxic to human gingival and periodontal ligament cells in *in vitro* conditions. However, the saliva nicotine concentrations seen in smokeless tobacco users can be cytotoxic in these conditions. Evidence of effects on cell attachment, cell proliferation and inflammatory mediator production suggested that effects could be seen at a wide range of nicotine concentrations but evidence was limited and often contradictory.

## Declaration of interests

The authors declare that there are no conflicts of interest in this study.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jdent.2019.05.030>.

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