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Non-irradiated campherquinone induces DNA damage in human gingival fibroblasts

Joachim Volk[a,1], Christina Ziemann[b,1], Gabriele Leyhausen[a], Werner Geurtsen[a,*]

[a] Clinic of Conservative/Preventive Dentistry and Periodontology, Hannover Medical School, 30625 Hannover, Germany
[b] Fraunhofer Institute of Toxicology and Experimental Medicine, 30625 Hannover, Germany

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ABSTRACT

Objectives. Camphorquinone (CQ) is cytotoxic in cell cultures. The mechanism of this toxic action, however, is not yet clearly understood. Aim of this investigation was to analyze the effects of non-irradiated CQ on intracellular formation of reactive oxygen species (ROS), intracellular glutathione (GSH) content, and the integrity of DNA in cultured primary human gingival fibroblasts (HGF).

Methods. Cells were exposed to CQ at concentrations ranging between 0.05 mM and 2.5 mM. Intracellular levels of ROS were detected by the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and GSH was determined by the fluorescent probe monobromobimane (MBBr). Genotoxicity was measured quantitatively by the alkaline comet assay. The cytotoxic effects of CQ were investigated by means of the fluorescent dye propidium iodide and the Cytotoxicity Detection Kit.

Results. CQ generated an increase of intracellular ROS, a depletion of intracellular GSH level, decreased cells' viability and total cell number dependent on the applied CQ concentration: 0.5–2.5 mM (ROS↑, GSH↓) and 0.125–2.5 mM CQ (cytotoxicity↑). Increased DNA damage was observed at all concentrations (0.05–2.5 mM, $p < 0.05$). The ROS-scavenger N-acetylcysteine (NAC) reduced CQ-induced ROS formation at CQ concentrations higher than 0.5 mM ($p < 0.05$).

Significance. Our data indicate that non-irradiated CQ induces oxidative stress, DNA damage and cytotoxicity as well in primary HGF.

1. Introduction

Camphorquinone (CQ) is the most important photoinitiator which is used in the vast majority of visible-light-curable dental resinous materials [1]. These resins usually contain about 0.2–1.5 wt% CQ [2] in combination with a co-initiator such as tertiary aromatic amines, e.g. N,N-dimethyl-p-toluidine (DMT), required by CQ for an efficient polymerization of (co)monomers [3,4]. CQ has two carbonyl groups with non-bonding electrons (Fig. 1). In one of the two groups an electron can be promoted to a short-lived, excited energy state by absorption of blue light. Wavelengths between 460 nm and 480 nm are used in clinical applications. In the presence of a tertiary amine, activated CQ and the amine can form free radical species, which promote the polymerization process [5].

CQ is not incorporated in the polymer network. Therefore, it may completely leach over time after polymerization. It was identified as one of the main leachables in extracts

* *Corresponding author.* Tel.: +49 511 532 4816; fax: +49 511 532 4811.
E-mail address: geurtsen.werner@mh-hannover.de (W. Geurtsen).
[1] These authors contributed equally to this work.
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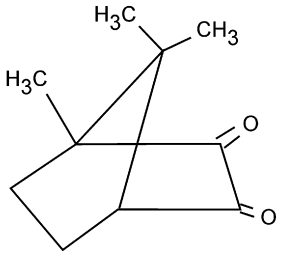

Fig. 1 – Chemical structure of camphorquinone (CQ).

of resin-based materials [6–11]. CQ reveals a moderate cytotoxicity compared to other photoinitiators [12–16] and most resin (co)monomers [6,17]. The mechanism of CQ cytotoxicity and its target structures at the cellular level are only partly understood. Recently, it has been shown that non-toxic concentrations of irradiated and non-irradiated CQ caused a rapid increase in intracellular reactive oxygen species (ROS) in human gingival fibroblasts (HGF), in a human epidermoid carcinoma cell line derived from a submandibular gland tumor [13], and in human pulp fibroblasts (HPF) [14,18]. Atsumi et al. reported that CQ produced more ROS than the aromatic ketones benzil, benzophenone, and 9-fluorenone in the absence or presence of reducing agents after visible-light (VL)-irradiation and, additionally, more radicals than 9-fluorene in the dark [13,14]. Subsequently, ROS may cause oxidative damage of the cells' macromolecules [19,20]. The production of ROS and the depletion of intracellular glutathione, one of the most important intracellular antioxidants, are two key factors resulting in apoptosis and/or pulp necrosis [21]. The oxidation of vital cell components like polyunsaturated fatty acids, proteins, nucleic acids, and to a lesser extent carbohydrates, may finally cause dysfunction or disease. It was also found that CQ induces cell cycle arrest followed by necrosis in HGF [16]. 'Sub-toxic' concentrations of CQ altered cell lipid metabolism, a critical pathway in maintaining cell membrane properties [22].

Pagoria et al. [23] reported that VL-irradiated CQ-induced single and double strand breaks in supercoiled plasmid DNA in a cell-free system. Previous studies using the prokaryotic *umu* test and the DNA synthesis inhibition test (DIT) with HeLa cells revealed that non-irradiated CQ is genotoxic and mutagenic [24]. This was not confirmed by Nomura et al. [25] using a bioluminescent bacterial genotoxicity test. These authors reported unclear results, because of the high cytotoxicity of those CQ concentrations which were also genotoxic. Taken together, only scanty and contradictory data about the genotoxic potency of CQ are available in the literature.

Therefore, it was objective of this investigation to analyze the cytotoxic and genotoxic potential of non-irradiated CQ in cultures of adherent primary HGF. The tested hypothesis was that CQ, even without visible-light-irradiation, is able to generate ROS, which may then cause DNA alterations. The capability of CQ to induce alkali labile sites and DNA strand breaks was assessed by the comet assay. Both types of DNA damage are related to ROS and/or delayed DNA repair in HGF. Since previous studies in our laboratory revealed that CQ cytotoxicity is caused by ROS generation, the effect of the ROS-scavenger N-acetyl-L-cysteine (NAC) was also analyzed.

2. Materials and methods

2.1. Cell cultures

Primary human gingival fibroblasts (HGF) were cultured from biopsies of healthy gingiva of permanent molars. Informed consent was obtained from the tissue donors according to the guidelines of the Institutional Review Board. The biopsies were stored at 4 °C for 24 h at most in Hank's balanced salt solution (HBSS, GIBCO BRL, Karlsruhe, Germany) supplemented with penicillin (200 $U \cdot mL^{-1}$), streptomycin (200 $\mu g \cdot mL^{-1}$), and amphotericin (5 $\mu g \cdot mL^{-1}$) (all from Biochrom KG, Berlin, Germany) prior to amplification. The tissue samples were placed into 25-cm^2 tissue culture flasks and grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5 $g \cdot L^{-1}$ glucose, 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 3.7 $g \cdot L^{-1}$ $NaHCO_3$, 100 $U \cdot mL^{-1}$ penicillin, and 100 mg mL^{-1} streptomycin (all from Biochrom KG, Berlin, Germany), supplemented with 10% fetal calf serum (FCS, Lonza, Verviers, Belgium) at 37 °C and 10% CO_2 in a humidified atmosphere. When outgrowth of cells was observed, the medium was replaced twice weekly until cells reached confluency. Cells were detached from the substrate by a brief treatment with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 0.02% EDTA) (Sigma, Deisenhofen, Germany) and cultured in 75-cm^2 tissue flasks until confluent monolayers were re-obtained. Early passages were frozen in liquid nitrogen. Cell viability (95–98%) was always analyzed before plating for experiments using trypan blue dye (Sigma, Taufkirchen, Germany) exclusion tests. All cultures were routinely tested for mycoplasma contamination by means of the mycoplasma detection kit Venor GeM (Minerva Biolabs, Berlin, Germany).

2.2. Treatment of cells with CQ

HGF from passages number 5–10 were seeded in 96-well plates (1×10^4 cells/well) and allowed to grow for 24 h. Subsequently, HGF were treated with various concentrations of CQ for 24 h (0.125–2.5 mM) and with 0.25–2.5 mM CQ alone and in combination with 0.5 mM NAC for 90 min and 3 h, respectively. Stock solutions (200-fold) of CQ (VOCO, Cuxhaven, Germany) and NAC (Sigma, Taufkirchen, Germany) were prepared in ethanol (Baker, Griesheim, Germany) and were freshly diluted in DMEM prior to each experiment. The final concentration of ethanol did not exceed 0.5% (v/v). In previous experiments it was found that an ethanol concentration of 0.5% (v/v) is non-toxic for HGF and has no effect on redox balance (data not shown). Cells incubated with fresh growth medium containing 0.5% ethanol (c1) and fresh growth medium without ethanol (c2) served as solvent controls (c1) and negative controls (c2).

The complete preparation of the used CQ solutions was done under dim green safe light and the cells were treated under dim room light to avoid the photoactivation of CQ.

2.3. Cell viability assays

2.3.1. Propidium iodide (PI) assay

HGF from passages number 5–9 were seeded in 96-well plates (1×10^4 cells/well) and allowed to grow for 24 h. After treat-