PHD Pharmaceuticals NV /
Ardoz Research BV

Effect of various reactive oxygen donors on DNA damage

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Chapter 1

Abstract

1. Abstract

Several studies published in scientific literature state damaging effects of peroxide type of reactive oxygen donors on cells and tissues. Hydrogen peroxide is widely used in professionally and self-administered products, in particular in tooth-whitening dentifrices. PHD Pharmaceuticals has initiated measurements to determine the effect of hydrogen peroxide and free radical generation on DNA. Tooth whitening products based on peroxide and one non-peroxide (Hydro-carbon-oxo-borate complex) based product (OT7051) were analysed. Measurements were executed at the Loma Linda and Indiana University in the USA, and at MUbio Products BV, affiliated with the Maastricht University, the Netherlands.

The conclusion is that the non-peroxide based product does not contain or produce hydrogen peroxide and generates a negligible amount of free radicals , that is significantly lower than that detected in hydrogen peroxide solutions or in the 10% carbamide peroxide product. These latter hydrogen peroxide donating products damage DNA, resulting in necrosis of the cells. The non-peroxide product did not show significant DNA damaging behaviour. This product can induce apoptosis, a physiological type of cell clearance, in a concentration dependent manner. Therefore, peroxides should be considered potentially dangerous when used in tooth whitening products.

Chapter

2 Peroxide content and free radical generation

Peroxide Content and Free Radical Generation by OT7051.
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The purpose of this study was to determine whether OT7051 contained peroxides and was capable of generating free radicals.

Peroxide content was analysed using a modified thiosulfate titration method. A titration calibration curve was constructed using freshly prepared H_2O_2 solutions. The results showed a linear relationship between the volume of sodium thiosulfate and the concentration of H_2O_2, validating the analytical procedures. Titration of a 10% carbamide peroxide gel showed 2.58% H_2O_2 content. No titration was needed for OT7051 indicating no detection of hydrogen peroxide in OT7051.

For the free radical generation assay, OT7051, a 35% H_2O_2 solution, and a 10% carbamide peroxide gel were analysed using an *in vitro* system that was added with FeEDTA using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as spin trapping agent. The data showed that H_2O_2 resulted in a dose- and time-dependent increase of DMPO-OH, indicating that it was a strong free radical-generating agent. The H_2O_2 solution resulted in an about two-fold increase in DMPO-OH production compared with the 10% carbamide peroxide gel; the difference was statistically significant ($p<0.01$).

A negligible amount of free radicals was detected only in the two highest doses (50 and 100 mg/mL) of OT7051; the values were >100-fold lower than that generated by the H_2O_2 solution ($p<0.01$) and >40-fold lower than the 10% carbamide peroxide gel ($p<0.01$).

Conclusion:

It is concluded that under the conditions of the studies, OT7051 Tooth Whitening Gel does not contain or produce hydrogen peroxide and it generates a negligible amount of free radicals that is significantly lower than that detected in a 10% carbamide peroxide bleaching gel.

Chapter 3

Effect of reactive oxygen donors on DNA damage

Introduction

Hydrogen peroxide compromises several cellular functions, the end result of which can be cell death by necrosis. While hydrogen peroxide may be injurious to tissue directly, secondarily derived oxidants such as hydroxyl radials will also contribute to tissue injury (reviewed in Walsh, 2000 and Lawrence et al, 2003). Hydrogen peroxide is a member of a family of related molecules termed reactive oxygen species. This family includes a number of radicals such as the superoxide (O_2.-) hydroxyl (HO.), peroxyl (ROO.) and alkoxyl (RO.) radicals.

Hydrogen peroxidase can damage cells via several mechanisms and delay cell division. In the presence of chloride ions, the action of peroxidase on hydrogen peroxide produces hypochlorous acid (HOCl), which acts at low molar concentrations (10-20 µmol/l) to damage proteins on cell membranes and destroy their function. In addition, hydrogen peroxide can diffuse through lipid membranes and once inside the cell it is able to react with iron, copper and other metallic ions to generate the highly reactive hydroxyl radical (HO.) and other oxidants. These substances initiate chain reactions of lipid peroxidation which cause decomposition of the phospholipids of cellular membranes and leakage of their destructive contents. The hydroxyl radical also damages the inner mitochondrial membrane, which can lead to the loss of viability of the cell. At the level of the individual cell, hydrogen peroxide can permeate cells rapidly and inhibit adenosine triphosphate (ATP) synthesis via both glycolytic and oxidative phosphorylation (mitochondrial) pathways. In the glycolytic pathway, damage is limited to the step involving glyceraldehyde-3-PO4 dehydrogenase (GAPDH). This results from both a direct attack on GAPDH and, indirectly, by a reduction in concentration of the GAPDH cofactor, nicotinamide adenine dinucleotide (NAD). This latter effect results from the activation of the enzyme, poly-adenosine diphosphate (ADP)-ribose polymerase, an enzyme involved in deoxyribonucleic acid (DNA) repair.

In particular, DNA can be damaged by hydrogen peroxide and other reactive oxygen species. The formation of DNA strand breaks leads to activation of poly-APG-ribose polymerase which in turn causes depletion of NAD and ATP, followed by calcium ion influx and eventually by cell lysis.

In culture studies, DNA damage in target cells has been reported at low concentrations of hydrogen peroxide (20-80 µmol/l) in several cell types. Strand breaks and base hydroxylation have been observed, resulting from the generation of hydroxyl radicals from hydrogen peroxide, in the presence of metallic ions.

DNA damage can result in either cell injury and necrotic cell death or mutations in the base sequence of DNA. The latter effects can lead to malignant transformations in cells cultured in the laboratory, and in vivo in T-lymphocyte deficient athymic mice.

The mutation frequency can increase by up to 30-fold over spontaneous background levels with increasing concentrations of hydrogen peroxide. These effects can be attributed to the potential for hydrogen peroxide, in the presence of metal ions, to cause breaks in DNA strands, resulting in gene loss or mutation. Strand breakage exposes DNA due to reduced protection from histones. Through DNA unwinding, exposure of additional sites for electrophilic attack near strand breaks can result in greater damage from other reactive oxygen species or from exogenous agents, unless the affected DNA is repaired rapidly and accurately.

Radical formation can induce cytotoxic effects in cells and tissues (Lawrence et al, 2003). Depending on the concentration and type of radical formed these effects may either result in apoptosis (physiologically programmed cell death) or necrosis.

Study set up

We have studied the effect of several tooth whitening preparations either based on hydrogen peroxide, carbamide-peroxide or hydro-carbon-oxo-borate complex (OT7051) on human epithelial cell lines with emphasis on the induction of DNA damage and the consequent induction of apoptosis or necrosis.

The tooth whitening preparations included :

1: Ardoz blanc (gel without whitener)
2: Ardoz Brite Brush Whitening gel (hydro-carbon-oxo-borate complex, OT7051)
3: Crest White Strips (hydrogen peroxide)
4: Colgate Simply White Night (hydrogen peroxide)
5: Colgate Simply White Day (carbamide peroxide)

Also a control experiment was included, with no additions at all.

The studies were performed in triplicate.

Materials & Methods

Cell lines tested

To investigate the effect of these whitening preparations on induction of apoptosis and/or necrosis in cultured cells, immortalized HaCat cells (Boukamp et al., 1988), as well as MCF-7 (human breast cancer cells) were used. These grow as adherent cells in EMEM (Minimum essential medium with Earle's salts, Gibco)

supplemented with 1% Glutamine, 1% non-essential amino acids, 0.1% gentamycin and 10% heat-inactivated fetal calf serum (HaCaT) or 10% heat-inactivated new born calf serum (MCF-7) in a humidified incubator at 37 ^{0}C and 5% CO_2. The cells were used in their exponential growth phase, and kept in a humidified incubator at 37 ^{0}C and 5% CO_2.

Determination of DNA-content

Cell suspensions fixed in methanol were incubated with 20 mg/ml propidium iodide (PI; Calbiochem, La Jolla, CA) for 15 min on ice in the dark. Thereafter they were subjected to flow cytometric quantification of their DNA content (see below).

M30 CytoDeath apoptosis assay

Apoptosis was detected and quantified as described previously (Leers et al., 1999). Briefly, methanol fixed cells were rinsed once in PBS. Appropriately diluted M30 CytoDeath antibody (Boeringer, Mannheim) was added to approximately 10^6 cells, resuspended in 100 μl PBS/BSA. After incubation for 1 h at room temperature, the cells were rinsed twice in PBS/BSA. For visualization FITC conjugated Fab_2 fragments of rabbit anti-mouse Ig (DAKO A/S, Glostrup, Denmark) antibody was added in a 1:10 dilution. After incubation for 45 minutes at room temperature samples were rinsed twice in PBS/BSA and the cells were finally resuspended in 0.5 ml cold PBS supplemented with 100 mg/ml RNAse (Serva) and 20 mg/ml propidium iodide (PI; Calbiochem, La Jolla, CA). The samples were allowed to stand for 15 minutes on ice in the dark before flow cytometric analysis. In the negative control the primary antibody was omitted.

The M30 CytoDeath-assay has recently been compared to other apoptosis-assays, and was found to be superior in HaCat cells (Galgon et al., 2000).

The flow cytometric analysis was essentially done as described below

Flow cytometric analysis

For flow cytometric analysis a FACSort (Becton Dickinson, Sunnyvale, CA) equipped with a single Argon ion laser was used. Excitation was done at 488 nm, and the emission filters used were 515-545 BP (green; FITC), and 600 LP (red; PI). A minimum of 10,000 cells per sample were analysed and data stored in list mode. FITC signals were recorded as logarithmic amplified data, while the PI signals were recorded as linear amplified data. For bivariate FITC/PI analysis no compensation was used.

Data analysis was performed with the standard Cellquest software (Becton Dickinson). As a standard procedure for all analyses, data were gated on pulse processed PI signals to exclude doublets and larger aggregates.

Results

The results of the three individual tests with the HaCat and MCF-7 cell lines can be summarized as follows :

a) The hydrogen peroxide and carbamide peroxide based products (Crest White Strips, Colgate products) induce significant DNA breakdown at low and high concentrations in both cell lines, as concluded from the sub-G1 DNA peaks observed in the flow cytograms shown in Figure 1. Ardoz Brite Brush showed much less DNA breakdown, even at the highest concentration.

b) The control incubations with only culture serum or addition of the Ardoz gel without the Ardoz withening preparation show similar basic levels of apoptosis induction. Both the low (1 mg/ml) and high (10 mg/ml) concentrations of the gel show approximately 3-5% apoptosis and virtually no necrosis, which is similar to the control experiments.
Typical example shown in Fig. 2A for HaCat.

c) Ardoz Brite Brush Whitening gel induces physiological cell death (apoptosis) in a fraction of both cell lines (example shown in Figure 2B for HaCat), but very low levels of necrosis. The percentage of cells induced to undergo apoptosis is concentration dependent and varies between 15-30%.

d) The Colgate preparations Simply White Night and Simply White Day induce massive necrosis at both 1 mg/ml and 10 mg/ml, as concluded from the breakdown of DNA and no induction of apoptosis seen in the M30/PI assay (Fig. 2C).

e) The Crest White Strip preparations induce both apoptosis and necrosis at the low concentration (1 mg/ml) and massive necrosis at high concentration (10 mg/ml).

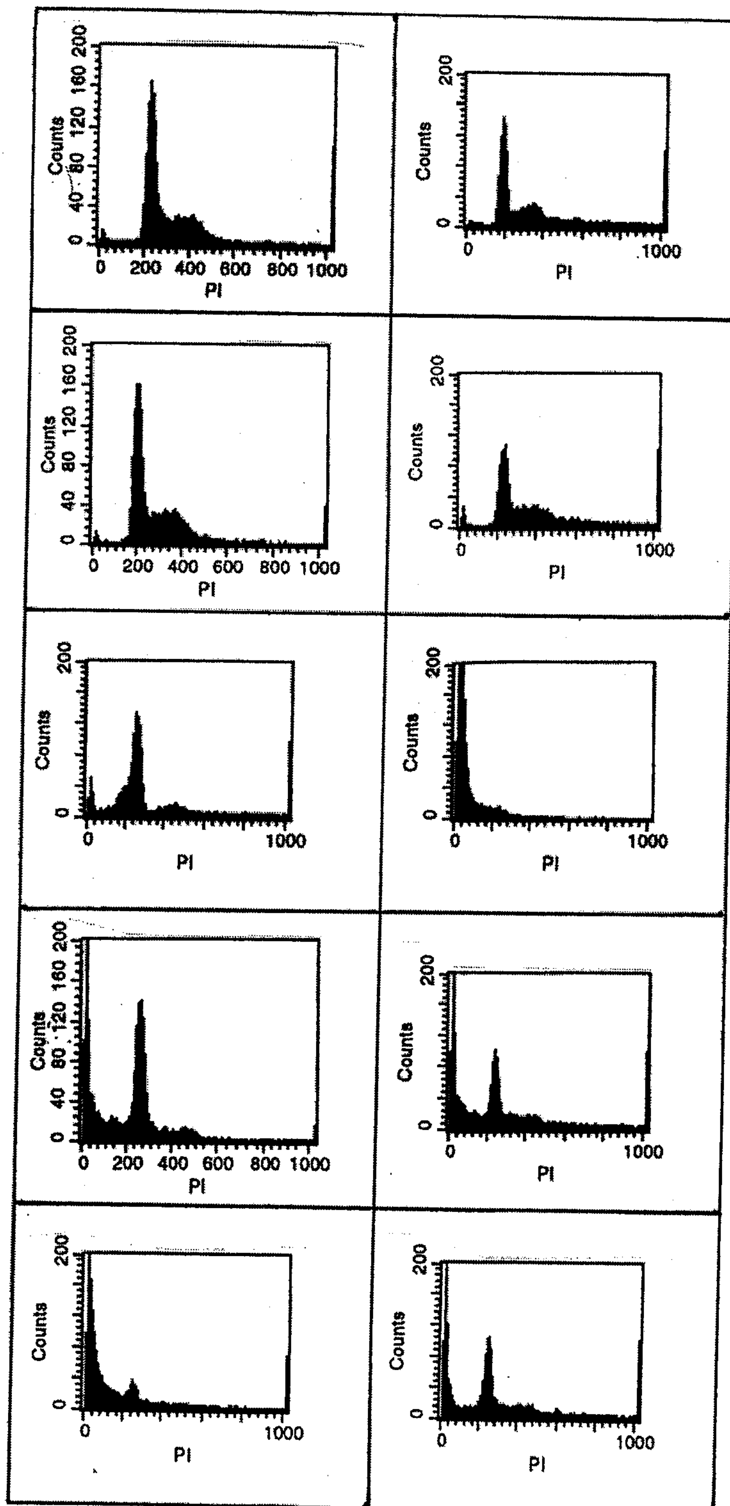

Figure 1

Ardoz Blanc (control)

10 mg/ml

Ardoz Brite Brush

10 mg/ml

Crest White Strips

10 mg/ml

Colgate Simply White Night

10 mg/ml

Colgate Simply White Day

10 mg/ml

HACAT

MCF-7

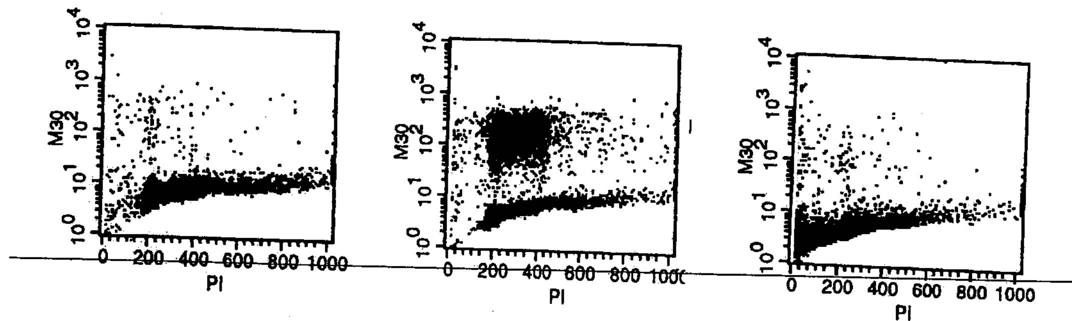

Figure 2A **Figure 2B** **Figure 2C**

Examples of induction of apoptosis and necrosis in HaCat cells by whitening preparations.

2A : Ardoz control gel. Very low (control) levels of apoptosis and necrosis.

2B : Ardoz Brite Brush (10 mg/ml) inducing apoptosis but not necrosis. The apoptotic cells are represented by the "cloud" of dots in the upper part of the diagram. These are M30 positive and exhibit a normal DNA content.

2C : Colgate Simply White Night inducing massive necrosis and DNA damage, but not apoptosis. The necrotic cells are represented by the "cloud" of dots in the lower left corner of the diagram. These are M30 negative and exhibit a decreased DNA content.

Chapter 4

Conclusion

4. Conclusion

It is concluded that under the conditions of the studies the OT7051 containing Ardoz Brite Brush Whitening Gel does not contain or produce hydrogen peroxide and generates a negligible amount of free radicals. These free radical concentrations are manifold lower than those detected in a 10% carbamide peroxide whitening gel and in hydrogen peroxide containing whitening products.

Ardoz Brite Brush gel therefore has a very limited effect on cellular DNA, in that it shows very limited DNA damage in cells treated with relatively high concentrations of the product. Hydrogen peroxide and carbamide peroxide based products showed a clear DNA-damaging effect, which resulted in cellular necrosis.

Chapter 5

List of references

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