

The *in vitro* Effects of Several Products of Dental Use on Tumor Cell Lines

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Chlorhexidine continues to be considered the gold standard in antiseptic treatment due to its high bactericidal activity. However, some studies reveal the fact that this compound may have some adverse effects both on tissues and cells at standard concentration frequently used in medical practice. Solutions Chlumsky and Walkhoff have a remarkable activity in the infection treatment, but the phenol and chlorophenol, respectively, are substances with a real toxic potential. The purpose of this study was to evaluate the effects of different products of dental use on viability of two tumor cell lines: A375 – human melanoma cells and B164A5 – murine melanoma cells. The dental solutions used in this study were: Gluco-Chex - Chlorhexidine digluconate, Chlumsky and Walkhoff solutions. In order to evaluate the effects of this solutions on cells viability, we tested 3 different concentrations for each solution according to the literature, as follows: Chlorhexidine digluconate (0.000025, 0.00025 and 0.0025%), Chlumsky (0.001, 0.01 and 0.1%) and Walkhoff (0.001, 0.01 and 0.1%). The cells were stimulated for 4 and 24 h and the viability was determined by the means of Trypan Blue and Alamar Blue assays.

Keywords: chlorhexidine, Chlumsky solution, Walkhoff solution, A375, B164A5

Chlorhexidine is a chemical compound with great bactericidal activity. It is used in dental practice for: (a) root canal disinfection, gingivitis controlling [1], (b) for the treatment of endodontic diseases and periodontal diseases [2], (c) its inhibitory effect on matrix metalloproteinase (MMP) [3, 4], and (d) its very good absorption and its effectiveness at low concentrations [5]. There were developed several *in vitro* studies on L929 fibroblasts, osteoblastic, endothelial or fibroblastic cell lines in order to evaluate the mechanism of action of this compound [5,6] which at the present moment is still incompletely elucidated.

The solutions Chlumsky and Walkhoff have in their composition camphorated phenol, and parachlorophenol, respectively, and are used to treat complicated infections. Studies of these solutions regarding their cytotoxicity are very limited and the ones that exist are focused on the antimicrobial activity [7-9].

The aim of the present study was to evaluate the effect of these three compounds used in dental practice, on two tumor cell lines viability: human melanoma cell line – A375 and murine melanoma cell line – B164A5.

Experimental part

Materials and methods

The cell lines used in this study were melanoma cell lines – A375 – human melanoma cell line and B164A5 – murine melanoma cell line. Both tumor cell lines were purchased from ECACC (European Collection of Cell Cultures) at passage no. 2.

The reagents used for cell culture were bought from Sigma Aldrich and were the following: culture media – Dulbecco's modified Eagle Medium (DMEM) high glucose–

4.5 g/L, foetal calf serum (FCS), penicillin/streptomycin solution, trypsin/EDTA solution, PBS (phosphate saline buffer), ethanol, trypan blue and Alamar blue. The test compounds test in the present study were: Chlorhexidine 2%, Chlumsky and Walkhoff solutions.

Cell culture

The two cell lines, A375 (human melanoma cell line) and B164A5 (murine melanoma cell line) were cultured in specific culture media – Dulbecco's modified Eagle Medium (DMEM) high glucose – 4.5 g/L (HEPES and L-glutamine) supplemented with 10% FCS and 1% penicillin (100UI/mL)/streptomycin (100 µg/mL). The cells were kept during the experiment in an incubator in standard conditions: 5% CO₂ and 37°C and were split every second day.

Trypan Blue cytotoxicity assay

One of the methods used in this study in order to evaluate the cells viability was the trypan blue assay. The mechanism of action is based on the capacity of this reagent to penetrate the membrane of the dead cells which become dark blue, whereas the viable cells are bright suggesting that were not affected by the action of trypan blue. The A375 and B164A5 cells (1X10⁶ cells/well) were cultured in a 6-well plate and the next day were stimulated with different concentrations of the three dental use test solutions: Chlorhexidine (0.000025, 0.00025 and 0.0025%), Clumsky (0.001, 0.01 and 0.1%) and Walkhoff (0.001, 0.01 and 0.1%) for 24 h. After the 24h stimulation, the cells were counted in the presence of trypan blue and the viability was calculated with specific formula [10].

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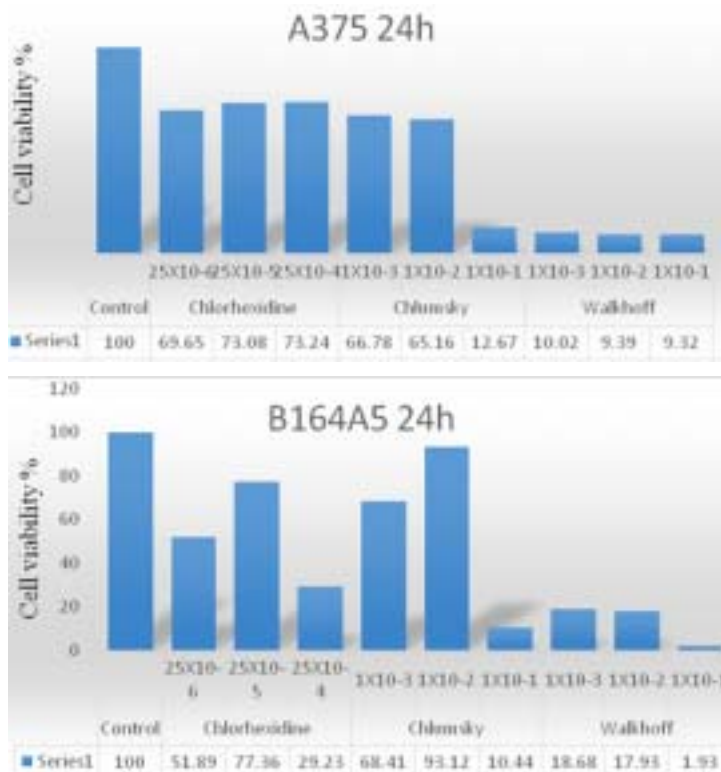


Fig. 1. The cytotoxic effect of the dental test compounds, chlorhexidine, Chlumsky and Walkhoff solutions, on human melanoma cells – A375. The cells were stimulated with 3 different concentrations/ test compound for 24h and then we tested the effect of this compounds on human melanoma cells viability using Alamar blue assay. As control we used A375 cells non-stimulated. The experiments were done in triplicate

Fig. 2. The cytotoxic effect of the dental test compounds, chlorhexidine, Chlumsky and Walkhoff solutions, on murine melanoma cells – B164A5. The cells were stimulated with 3 different concentrations/ test compound for 24h and then we tested the effect of this compounds on human melanoma cells viability using Alamar blue assay. As control we used B164A5 cells non-stimulated. The experiments were done in triplicate

Alamar blue cytotoxicity assay

Another assay used for the evaluation of the effects of the three test compounds on the viability of the A375 and B164A5 cells was Alamar blue cytotoxicity assay, an established technique for cytotoxicity and cell proliferation studies. The principle of this method consists in the capacity of the viable cells to reduce resazurin to resofurin which is a molecule that produces a red fluorescence that can be quantified and the results obtained are quantitative [11].

A375 and B164A5 cells (1×10^4 cells/well) were cultured in 96-well plates and incubated over night in specific culture media. The next day the culture media was replaced with new culture media that contained different concentrations of the test compounds: Chlorhexidine (0.000025, 0.00025 and 0.0025%), Chlumsky (0.001, 0.01 and 0.1%) and Walkhoff (0.001, 0.01 and 0.1%) for 4 and 24 h. After the 4h/24h of stimulation it was added a volume of 20 μ L Alamar blue/well (10% of the total media volume) and the cells were incubated for 4-5h. Subsequently the absorbance was read using a spectrofotometer at two different wavelengths: 492 and 630 nm (Stat Fax 2100 Awareness Technology Inc).

Scratch assay

This technique is a wound healing-like method developed *in vitro* and it is used to determine the effect of the test compounds on the migration of the tumor cells.

A375 and B164A5 cells (2×10^5 cells/well) were cultured in 12-well plates and were let to adhere to the plate for 24h (until the confluence was at 90%). The media was removed and there were done scratches using a pipette tip of 10 μ L. The detached cells were washed with PBS and the cells were stimulated with different concentrations of test compounds: Chlorhexidine (0.000025, 0.00025 and 0.0025%), Chlumsky (0.001, 0.01 and 0.1%) and Walkhoff (0.001, 0.01 and 0.1%) for 24 h. There were taken pictures at different time points: 0, 3 and 24h using a camera (Optika Microscopes Optikam Pro Cool 5 and Optika View).

Results and discussions

The present study was proposed in order to evaluate the effect of three of the most used products in the dental practice, chlorhexidine, Chlumsky and Walkhoff solutions on the viability of two cancer cell lines: A375 – human melanoma cells and B164A5 – murine melanoma cells. We used 3 different concentrations for each compound and the cells were stimulated for 4 and 24h. The cytotoxic effect of the compounds was checked by the means of Trypan blue and Alamar blue cell viability assays.

In the case of human melanoma cell lines – A375, our results indicate that the highest cytotoxic effect (approx. 10% viable cells) was observed after the stimulation with Walkhoff solution, even at the lowest concentration used in the study (0.001%). A cytotoxic effect was also observed at the highest concentration of Chlumsky solution (0.1%). Our data showed that A375 cells were less sensitive to chlorhexidine solution effects, the viability of the cells being decreased only with (25-30%) as compared to the other solution when we obtained a 10% viability (fig.1). Similar results were obtained when we checked the viability using Trypan blue assay (data not shown).

When we stimulated the cells for 4h with the test solutions, it was obtained a similar cytotoxic effect, but the results weren't as significant as the ones obtained after 24h stimulation.

The stimulation of the murine melanoma cells B164A5 with Walkhoff solution led to similar results as the ones observed for the human melanoma cells, a significant percent of death cells being observed even at the lowest concentration. Our results indicate that B164A5 were affected by chlorhexidine solution, too, the highest cytotoxic effect being observed at the highest concentration. An unexpected result was obtained in the case of the middle concentrations of both chlorhexidine (0.00025%) and Chlumsky (0.01%) solutions, when the viability of cells was around 80%. The highest concentration of Chlumsky solution (0.1%) induced cell death in a

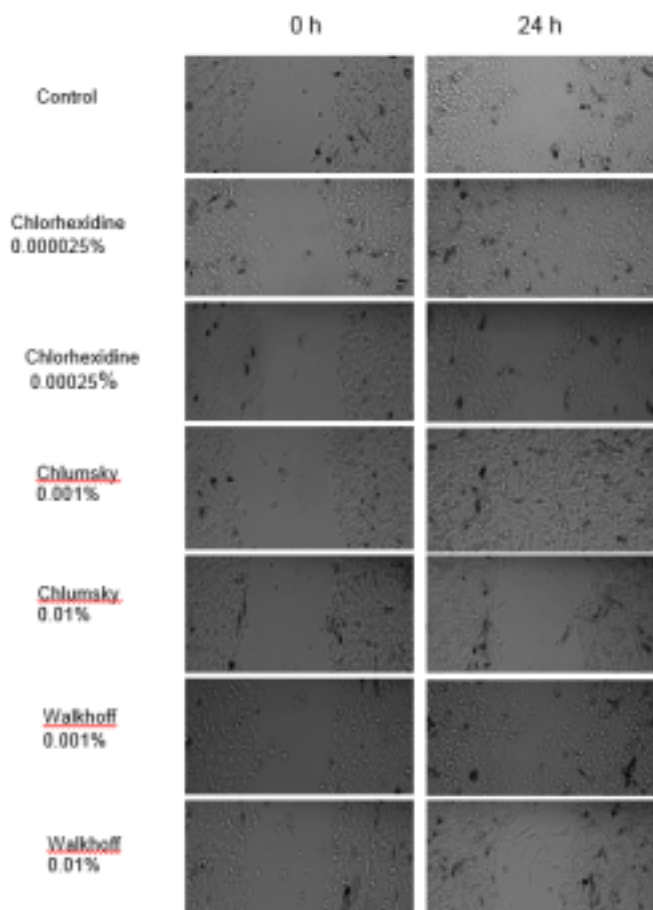


Fig. 3. The inhibitory effect of Chlorhexidine, Chlumsky and Walkhoff solution on the capacity of migration of murine melanoma cells - B164A5

significant percentage (aprox. 90%) (fig. 2). Similar data were obtained for Trypan blue assay (data not shown).

It is well-known that tumor cells are characterized by a high capacity of migration during second tumors development. Taken into account the results obtained regarding the cytotoxic effects of the test compounds, we decided to test the effect of these solutions on the capacity of tumor migration by using Scratch assay. Eloquent results were obtained for the murine melanoma cells and these data will be presented further in figure 3.

Taken into account the results that we obtained for the cell viability assay, we tested the effects of these compounds on cells migration by using only the concentrations that not induced a significant cell death of the cells (the lowest and the medium concentrations: 0.000025 and 0.00025% - for Chlorhexidine and 0.001 and 0.01% - for Chlumsky and Walkhoff solutions). As it can be seen from figure 3, an inhibitory effect on tumor cells migration was detected only in the case of the highest concentrations used in this experiments, whereas the lowest concentrations were associated with a stimulatory effect of cell migration capacity. These data suggest the fact that the test solutions were able not only to induce cell death, but also to inhibit tumor cell migration, the effects being dose-dependent.

Conclusions

Our results indicate that Chlorhexidine digluconate solution 2% induced in a small percentage the cell death of the tumor cells. Chlumsky solution at the highest concentration used in the study affected seriously the viability of both tumor cell lines. A high cytotoxic effect in case of both A375 and B164A5 cells was observed after stimulation with Walkhoff solution (all three concentrations), changes in cells shape being detected after first hours post-stimulation. The effect of the test compounds on the migratory capacity of tumor cells was dose-dependent: the lowest concentrations had a stimulatory effect, whereas the highest concentrations were associated with an inhibitory effect. Further studies are required in order to elucidate the underlying mechanism of action of these dental solutions.

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References

1. T.F. GRASSI, E.A. CAMARGO, D.M.F. SALVADORI, M.E.A. MARQUES, D.A. RIBEIRO, DNA damage in multiple organs after exposure to chlorhexidine in Wistar rats, *International Journal of Hygiene and Environmental Health* 210, 2007, p. 163-167
2. M.R. CARRILHO, R.M. CARVALHO, E.N. SOUSA, J. NICOLAU, L. BRESCHI, A. MAZZONI, L. TJADERHANE, F.R. TAY, K. AGEE, D.H. PASHLEY, Substantivity of chlorhexidine to human dentin, *Dental Materials* 26, 2010, p. 779-785
3. M. EKAMBARAM, C.K.Y. YIU, J.P. MATINLINNA, N.M. KING, F.R. TAY, Adjunctive application of chlorhexidine and ethanol-wet bonding on durability of bonds to sound and caries-affected dentine, *Journal of Dentistry* 42, 2014, p. 709-719
4. R. STANISLAWCZUK, A. REIS, A.D. LOGUERCIO, A 2-year in vitro evaluation of a chlorhexidine-containing acid on the durability of resin-dentin interfaces, *Journal of Dentistry* 39, 2011, p. 40-47
5. N. SALIM, C. MOORE, N. SILIKAS, J. SATTERHWAITE, R. RAUTEMAA, Chlorhexidine is a highly effective topical broad-spectrum agent against *Candida* spp., *International Journal of Antimicrobial Agents* 41, 2013, p. 65-69
6. G. FARIA, C.R.B. CARDOSO, R.E. LARSON, J.S. SILVA, M.A. ROSSI, Chlorhexidine-induced apoptosis or necrosis in L929 fibroblasts: A role for endoplasmic reticulum stress, *Toxicol Appl Pharm* 234 (2009) 256-265
7. M. GIANELLI, F. CHELLINI, M. MARGHERI, P. TONELLI, A. TANI, Effect of chlorhexidine digluconate on different cell types: A molecular and ultrastructural investigation, *Toxicol in Vitro* 22 (2008) 308-317
8. M.Y. SHURRAB, Antimicrobial Efficiency of Some Antiseptic Products on Endodontic Microflora Isolated from Gangrenous Pulp Tissue, *J Contemp Dent Pract* 7(4) (2006) 1-8
9. F.B. DE ANDRADE FERREIRA, S.A. TORRES, O.P. DA SILVA ROSA, C.M. FERREIRA, R.B. GARCIA, M.C. MARCUCCI, B.P.F.A. GOMES, Antimicrobial effect of propolis and other substances against selected endodontic pathogens, *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 104, 2007, 709-716
10. BYSTROM, A., CLAEISSON, R., SUNDQVIST, G., The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals, *Endod Dent Traumatol* 1985, 1, 170-175
11. PHELAN, M., C., Basic Techniques for Mammalian Cell Tissue Culture. In: *Current Protocols in Cell Biology*, Phelan MC (ed.), New York: John Wiley & Sons, Inc., 1998.
12. *** AlamarBlue® Technical Datasheet. 2002, AbD Serotec Ltd.

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