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DiaClean® water technology for the postharvest preservation of citrus fruits causes no cytotoxic effect after air-drying and storage

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Abstract

Citrus fruits are one of the most important sources of income in many countries and are widely cultivated in the Mediterranean area. During postharvest packing, storage and transportation, citrus fruits can be infestated by a variety of diseases such as mold infection. Thus, the control of postharvest disease is essential for the quality and shelf-life of citrus fruits.

In this present study, the DiaClean® water technology which is based on the electrolysis of a 1.25% $NaHCO_3$ solution with boron-doped diamond electrodes as an alternative to the treatment of citrus fruits with fungicides, was used for fruit preservation. Aliquots of the total mixtures of compounds generated at different stages of the process were used to investigate their cytotoxic effects as an indicator for the evaluation of human health and environmental risks. Cultured connective tissue fibroblasts (cell line L-929) were used for the assays and cell vitality was checked by measurement of the enzymatic activity of mitochondrial dehydrogenases within the cells.

The results clearly demonstrate that the use of electrolyzed $NaHCO_3$ in the preservation of citrus fruits is very effective in the acute stage of electrolysis; the occurrence of antimicrobial and cytotoxic compounds is limited to the liquid stages of the process. After air-drying, no cytotoxic effect was observed which might be harmful to human health and the environment.

Abbreviations: BDD: Boron-doped diamond electrodes, ROS: reactive oxygen species

Introduction

Citrus fruits are one of the most important sources of income in many countries and are widely cultivated in the Mediterranean area. During postharvest packing, storage and transportation, which may take several weeks, citrus fruits can be infestated by a variety of diseases. Among these, the disease caused by a green mold *Penicillium digitatum* is one of the most serious ones and might cause up to 90% of product losses [1]. Thus, the control of postharvest disease is essential for the quality and shelf-life of citrus fruits.

The use of various fungicides and herbicides such as imazalil (fungicide), thiabendazole (fungicide and herbicide), pyrimethanil (fungicide) and others has minimized postharvest infestation [2], but the extensive application has led to resistant strains, which decrease their effectiveness. In addition, human health and environmental risks are associated with the use of such fungicides [3]. As a matter of fact, alternative strategies which combine effectiveness and low health and environmental risks have been developed during the last years.

One of the very promising technologies for this problem seems to be the use of electrolyzed water which has been tested successfully in food industrial processes [4] and against postharvest decay of fresh fruits and vegetables [5-7]. Thus, the use of Boron Doped Diamond (BDD) electrodes for electrolysis of citrus fruit wash water proved to be effective and could be increased by the addition of $NaHCO_3$ to the water prior to electrolysis [8-10]. The technology is based on the idea of using diamond as an electrode material in electrochemistry. Especially BDD electrodes have shown outstanding electrochemical properties in the oxidation of organic and inorganic compounds [11]. The reactivity can be mainly associated with the production of hydroxyl radicals [12] or superoxide anion radicals [10]. However, the detailed mode of action is currently not well understood as well as its possible potential to generate long-lasting compounds which might be harmful for human health and environment.

In this present study, the DiaClean® water technology which is based on the electrolysis of a $NaHCO_3$ solution with BDD electrodes, was used for citrus fruit preservation. Aliquots of the total mixtures of compounds generated at different stages of the preservation process were used to investigate their effect on vitality of cultured connective tissue fibroblasts, and thus, to evaluate possible health and environmental risks. To our knowledge, the cytotoxicity of the subsequent process stages has not been examined before.

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Materials and methods

Preparation of electrolyzed water according to DiaClean® technology

2.5 L of supply water (15°C) were poured into the reservoir of the DiaClean® Kit equipment (WaterDiam France SAS; Franken, France) and electrolyzed with a current flow of 4.0 A, and a volumetric flow rate of 200 L/h. The voltage was 25 V during electrolysis. The water was cooled during electrolysis by a cooling coil with circulating cold water to avoid a temperature increase above 20°C. After 30 min a total charge of 0.8 Ah/L was delivered to the water which is similar as described in the report of Fallanaj *et al.* [10]. An aliquot of the electrolyzed water was filtered sterile with a 0.45 µm porous membrane filters and added to the cell cultures.

Preparation of electrolyzed 1.25% aqeous $NaHCO_3$ solution

2.5 L of supply water containing 1.25% (w/v) $NaHCO_3$ solution (31.25 g $NaHCO_3$ dissolved in 2.5 L of supply water) were subjected to electrolysis by the DiaClean® Kit equipment as described above with a current flow of 4.0 A and a volumetric flow rate of 200 L/h. The voltage was 8 V during electrolysis. After 30 min a total charge of 0.8 Ah/L was delivered to the $NaHCO_3$ solution which was then filtered sterile and added to the cell cultures.

Overview of all experiments with electrolyzed water and $NaHCO_3$ solution

Based on the preparations of electrolyzed water and $NaHCO_3$ solution as described above, we conducted the following experiments to come to a closer understanding at which stages of the process a cytotoxic effect might occur:

1 Cytotoxicity of supply water ± electrolysis

1.1 Supply water

1.2 Supply water electrolyzed with 0.8 Ah/L directly after electrolysis

1.3 Supply water electrolyzed with 0.8 Ah/L and after storage for 15 min at room temperature

2 Cytotoxicity of 1.25% $NaHCO_3$ solution ± electrolysis

2.1 1.25% $NaHCO_3$ solution before electrolysis

2.2 1.25% $NaHCO_3$ solution directly after electrolysis with 0.8 Ah/L and after storage for 15 min, 2 h and 5 days at room temperature

2.3 1.25% $NaHCO_3$ solution directly after electrolysis with varying total charges of 0.05 - 0.1 - 0.2 - 0.4 - 0.6 - 0.8 Ah/L

3 Cytotoxicity of citrus fruits after treatment with electrolyzed 1.25% $NaHCO_3$ solution

Untreated/unpreserved BIO citrus fruits (oranges, lemons and grapefruits – fruits were selected for absence of any disease or surface wounding) were carefully prewashed with supply water of 40 °C, dipped for 45 to 60 seconds in freshly prepared electrolyzed $NaHCO_3$ (total charge of 0.8 Ah/L), air-dried at normal daylight for 24 h and stored at room temperature for another 24 h. Then, fruits were washed in 25 ml of supply water (20 °C) for each fruit and the sterilized washing solutions were used for the cytotoxicity tests.

Cell culture and culture conditions

L-929 (mouse connective tissue fibroblasts; ACC 173; Leibniz-Institut - Deutsche Sammlung von Mikroorganismen und Zellkulturen; Braunschweig, Germany); internal passage 93-97; recommended for assessment of in vitro cytotoxicity according to EN ISO 10993-5: 2009. Cells were routinely cultivated as mass cultures in an incubator at 37 °C with a moist atmosphere of 5% CO_2 and 95% air. Culture medium was RPMI 1640 supplemented with 10% fetal bovine serum and 100 Units/ml of penicillin and 100 µg/ml of streptomycin.

Cytotoxicity assay

The investigations were performed according to EN ISO 10993-5: 2009 (tests for in vitro cytotoxicity) with some modifications.

L-929 cells were taken from 80 to 90% confluent mass cultures and seeded into 96-well plates at a density of 5,000 cells/well in 200 µL of culture medium. 24-48 hours after seeding, cells were exposed to increasing concentrations (up to 50%) of sterile supply water as solvent control and the solutions from the various experimental designs as described in detail. After 3 days of continuous exposure, cell cultures were examined morphologically and cell vitality was measured by the activity of mitochondrial dehydrogenases within the cells using XTT (Xenometrix AG; Allschwil, Switzerland).

XTT is the sodium salt of 2,3-bis[2-methoxy-4-nitro-5-sulfopheny]-2H-tetrazolium-5-carboxyanilide and has a yellowish colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring of XTT yielding orange formazan crystals which are soluble in aqueous solutions. The intensity of the resulting orange solution correlates directly with cell vitality and metabolic activity [13,14].

For measurement of cell vitality after exposure, culture medium ± added solutions were removed by aspiration and 200 µL/well of fresh culture medium containing 10% XTT were added. After another 120 minutes of incubation at 37 °C with the tetrazolium dye, optical density per well was determined at 450 nm and 690 nm as a difference measurement using a double-wavelength BioTEK Elx 808 ELISA reader. Optical densities at different extract dilutions was compared with corresponding reagent controls and relative cell vitality was calculated as mean value ± standard deviation. All experiments were done either in triplicate or quadruplicate. Statistical analysis was done by using the two-tailed Wilcoxon-Mann-Whitney U-test.

Results and discussion

The addition of supply water caused a concentration dependent reduction in cell vitality of connective tissue fibroblasts after a continuous exposure time of 3 days due to the decreased osmolarity of the culture medium (Figure 1). Therefore, further experiments were only conducted with a maximum of added water of 50 vol% and all data were expressed as relative values in comparison to untreated controls containing the same amount of supply water without any treatment.

Electrolyzed water with a total charge of 0.8 Ah/L had no effect on the vitality of cultured fibroblasts (Figure 2) neither directly after electrolysis nor after 15 min of storage at room temperature indicating that no cytotoxic compounds have been generated which would be able to reduce the incidence of green mold on the surface of unpreserved citrus fruits. In previous investigations, electrolyzed water has been tested for its use in industrial food processes [4] and against postharvest decay of fresh fruits and vegetables [5-7]. However, the acidification or addition of various organic and inorganic salts to the water during electrolysis enhanced its antimicrobial efficacy. Especially the introduction of $NaHCO_3$ proved to be very effective in the preservation of citrus fruits [15] and it was shown to be a potent inhibitor of *Penicillium* rots [8,9].

In accordance to the effectiveness of electrolyzed $NaHCO_3$ as demonstrated by Fallanaj *et al.* [8-10] for *Penicillium* inhibition, were the data on cytotoxicity obtained with 1.25% $NaHCO_3$ in supply water as presented here. While the exposure of cultured connective tissue fibroblasts to 1.25% $NaHCO_3$ in supply water prior to electrolysis had no influence on cell vitality after 3 days (Figure 3A), the electrolyzed $NaHCO_3$ (total charge of 0.8 Ah/L) directly after electrolysis and after a time period up to 5 days of storage resulted in a marked cytotoxic effect (Figures 3B-3F). However, the cytotoxicity slowly decreased with storage time, but was still statistically significant at test concentrations > 10 vol% after 5 days of storage. This indicates that electrolysis of $NaHCO_3$ resulted in the generation of cytotoxic compounds which seem to be responsible for the antimicrobial efficacy of this process in citrus fruit preservation. In order to test whether a variation of total charge applied to 1.25% $NaHCO_3$ in supply water might influence the observed cytotoxic effect, a total charge ranging from 0.05 Ah/L to 0.8 Ah/L was used and cytotoxicity was examined. As shown in Figure 4, electrolysis of $NaHCO_3$ generated cytotoxic compounds in a charge-dependent manner.

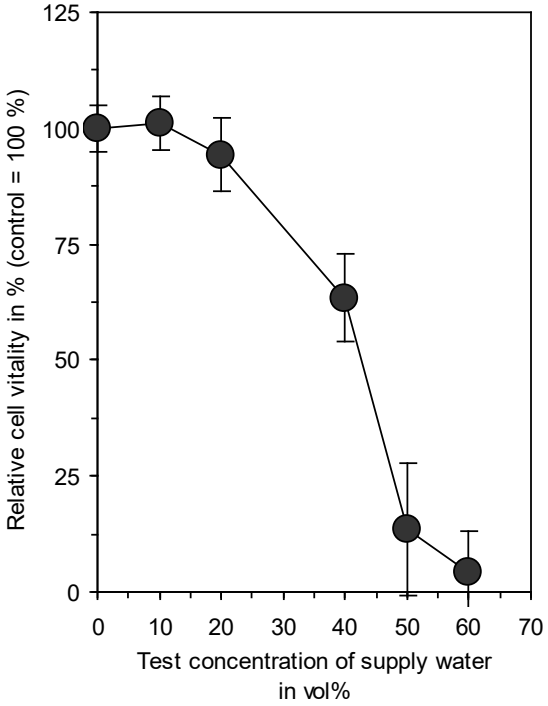

Figure 1. Dose-dependent reduction of vitality of cultured connective tissue fibroblasts (L-929) with increasing concentrations of supply water present in the culture medium by reduction of osmolarity. Exposure time was 3 days. Data represent mean value ± standard deviation (n = 3).

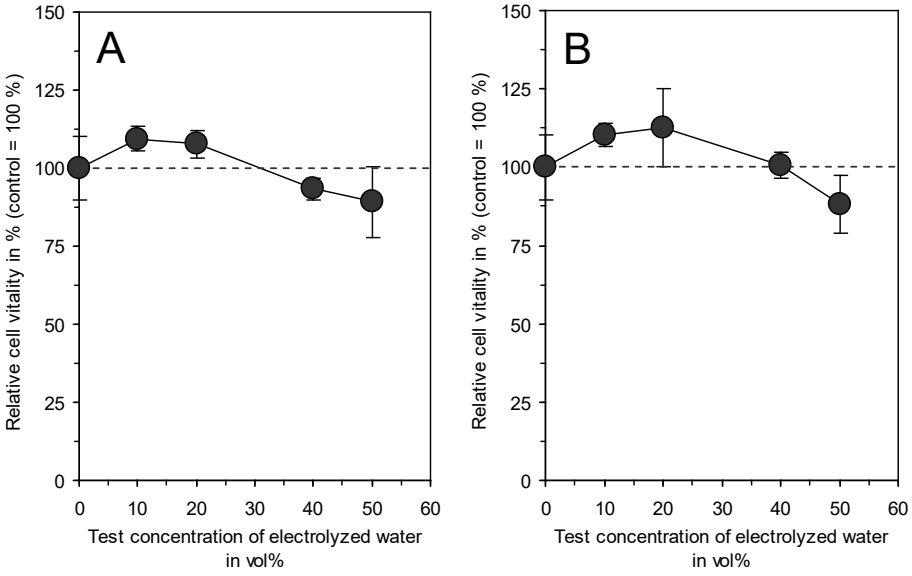

Figure 2. Effect of electrolyzed water (total charge of 0.8 Ah/L) directly (A) and after 15 min of storage at room temperature (B) on vitality of cultured connective tissue fibroblasts (L-929) after 3 days of continuous exposure. Both experimental setups do not show a significant difference between treated cells and corresponding controls. Data represent mean value ± standard deviation (n = 3).

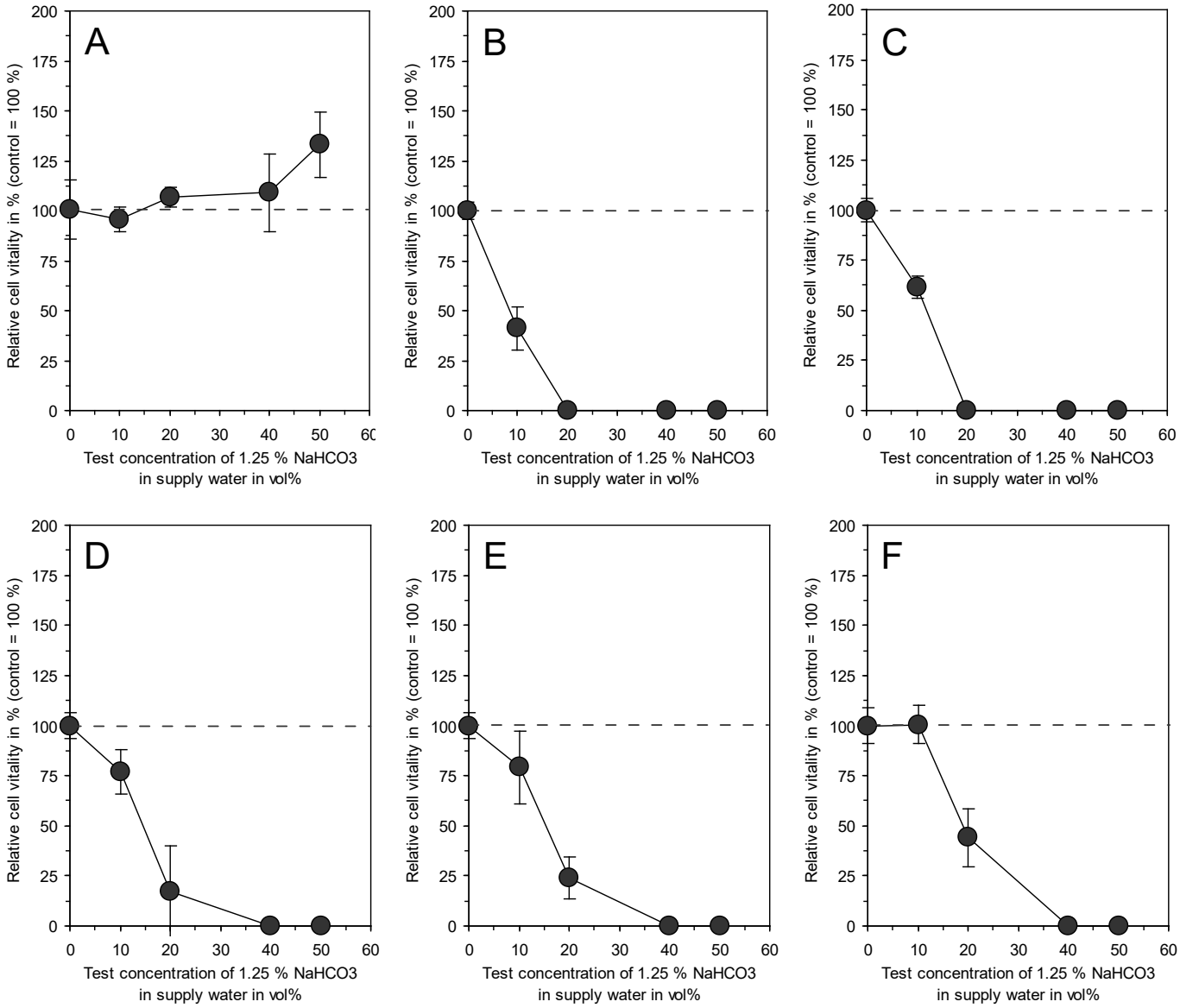

Figure 3. Effect of 1.25 % $NaHCO_3$ dissolved in supply water before electrolysis (A), directly after electrolysis with a total charge of 0.8 Ah/L (B) and after a storage at room temperature for 15 min (C), 2 h (D), 12 h (E) and 5 days (F) on vitality of cultured connective tissue fibroblasts (L-929) after 3 days of continuous exposure. Note that the storage time slowly reduces the cytotoxicity of the electrolyzed $NaHCO_3$, but is still prominent at concentrations higher than 10 vol% after 5 days. Data represent mean value ± standard deviation (n = 3).

Both results clearly indicate that electrolysis of 1.25% $NaHCO_3$ resulted in the generation of cytotoxic and antimicrobial compounds. Although the process stages are not completely understood, there are some reports on the generated compounds. Jeong *et al.* [16] reported that the electrochemical production of reactive oxygen species (ROS) could be responsible for microbial inhibition. Moreover, the BDD electrode itself might also produce ROS including superoxide anion radical and hydrogen peroxide [10,17].

In the final experimental series of this study, it was tested whether the application of freshly prepared electrolyzed $NaHCO_3$ to unpreserved fruits with subsequent air-drying and storage might also result in cytotoxic and harmful compounds. The air-dried surface of oranges, lemons and grapefruits was somewhat powdery and could be easily washed off and collected. This washing extract showed no longer a cytotoxic effect in the test assays at all concentrations in comparison to the controls (Figure 5). This suggests that the primarily generated cytotoxic compounds of the electrolyzed $NaHCO_3$ as depicted in Figures 3 and 4, which are necessary to reduce the incidence of mold, are no longer present after air-drying of the surface. In addition, the experiments also demonstrated an increased cell vitality for all washing extracts at the highest test concentration, probably giving an indication that the treated fruits might be preserved in a way which does not only enhance their shelf-life, but might also result in an improved fruit quality.

Conclusion

In conclusion, the use of electrolyzed $NaHCO_3$ in the preservation of citrus fruits is very effective in the acute stages of electrolysis; the occurrence of antimicrobial and cytotoxic compounds is limited to the liquid stage of the process. After air-drying, no cytotoxic effect was observed which might be harmful to human health and the environment.

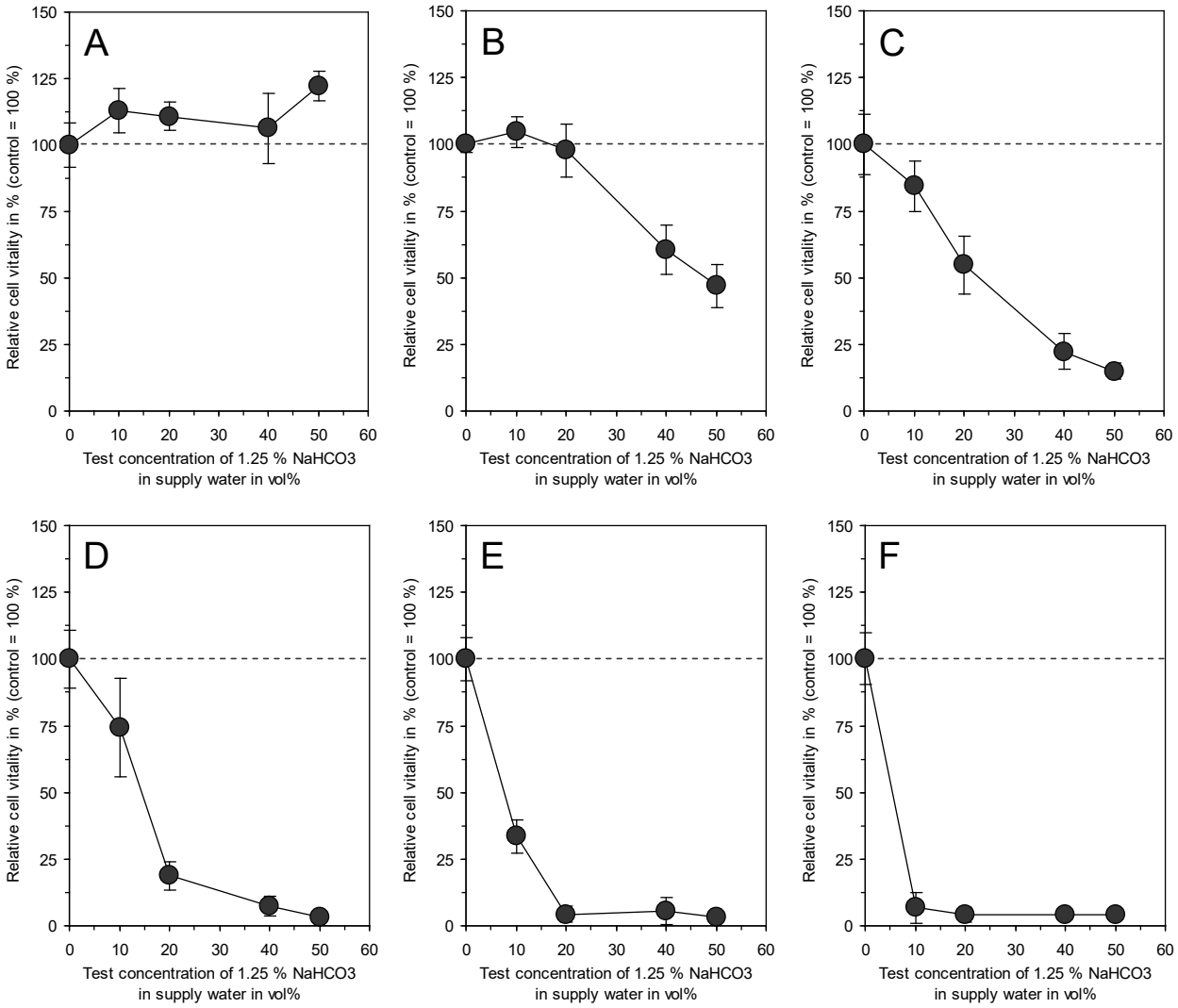

Figure 4. Effect of 1.25 % $NaHCO_3$ dissolved in supply water before electrolysis (A) and directly after electrolysis with a total charge of 0.05 Ah/L (B), 0.1 Ah/L (C), 0.2 Ah/L (D), 0.4 Ah/L (E) and 0.6 Ah/L (F) on vitality of cultured connective tissue fibroblasts (L-929) after 3 days of continuous exposure. Note that electrolysis of $NaHCO_3$ obviously generates cytotoxic compounds in a charge-dependent manner. Data represent mean value ± standard deviation (n = 3).

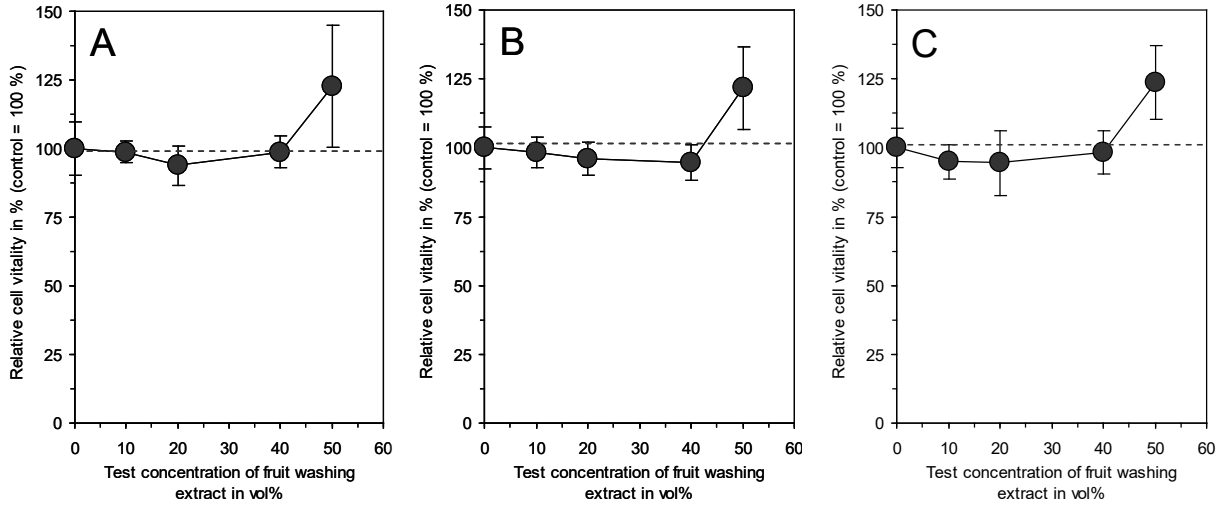

Figure 5. Effect of the surface washing extracts of orange (A), lemon (B) and grapefruit (C) on vitality of cultured connective tissue fibroblasts (L-929) after a continuous exposure time of 3 days. Note that no one of the washing extracts is cytotoxic and does not reduce cell vitality in comparison to untreated controls. Moreover, the highest test concentration increases cell vitality in all extracts. Data represent mean value ± standard deviation (n = 4).

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