Department of Biochemistry and Molecular Biology

Faculty of Biology

Ionfilter.

In vitro study on the cytotoxic and antioxidant effect of a hydrogen water on human cell lines.

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Objective

The purpose of this study is to determine the possible effects on vitality caused by electrolysed water and the presence of hydrogen gas in it, as well as the cell antioxidant activity by using two human cell models.

Experimental design:

To study the effect of electrolysed water we have used a healthy cell line such as ARPE-19 (ATCC® CRL-2302™, human retinal pigment epithelial cells), and a tumour cell line such as HeLa cells (ATCC® CCL-2™, human adenocarcinoma epithelial cells, specifically from cervical cancer).

Electrolysed water is obtained from purified water just before being used. This water is produced with a milli-Q system (Merck KGaA, Darmstadt, Germany). Water is introduced into the HYDRON compact hydrogenating system (supplied by the company IONFILTER), which enriches water with hydrogen by means of an electrolysis process. A continuous recirculation process is carried out for 10 minutes, thus obtaining hydrogen water with an electrochemical potential of -790 ± 5 mV, which is measured with a portable ORP measuring device (Milwaukee Electronics Kft., Szeged, Hungary). To work with cell lines, this water is immediately sterilised using filtration: a filter of 0.22 µm under pressure. Its dilution in concentrated PBS (10X) does not change its potential, but it is slightly reduced to -650 ± 10 mV in the culture media.

Experimental procedure:

Cell cultures

Each of the cell cultures used in the study is cultured and kept in the appropriate growth medium. For the ARPE-19 cell line, the cells grow and are maintained in DMEM-F12 (Biological Industries, Kibbutz Beik Haemek, Israel), and are also supplemented with 10% of foetal bovine serum, 1% glutamine 200mM, 100 UI/mL penicillin and 100 µg/mL streptomycin. For the HeLa cell line, the medium used is DMEM (Biological Industries, Kibbutz Beik Haemek, Israel), which is also supplemented with serum, glutamine and antibiotics.

Both cell lines are maintained at 37°C inside an incubator (NUAIRE, Fernbrook Lane N Plymouth, USA) with a humidified atmosphere and 5% CO_2. Cells are left to grow up to 80% of convergence in a 75 cm^2 polystyrene flask. Cells grow in an adherent form and, after an enzymatic treatment with trypsin, they are separated to be able to plate them in microplates at the appropriate density for the study. In this step, cell viability and the assessment of the number of cells are determined with an exclusionary assay of the trypan blue colouring, by counting the non-stained cells in the automatic cell counter Luna (Logos Biosystems, Anyang-si, Gyeonggi-do, South Korea).

Cell viability assay

Each cell line is plated in microplates of 96 wells with enough cell density as to ensure an exponential growth, without reaching saturation. The density of ARPE-19 cells is of $7.5 \cdot 10^4$ cells/cm^2 and of $1.5 \cdot 10^4$ cells/cm^2 for HeLa cells. They are incubated during 24h at 37°C to enable adherence on the well surface. Each study time (0, 24, 48 and 72h) and each cell line are plated in different microplates.

We work with four different media:

1. MEDIA 0: Supplemented DMEM-F12 or DMEM media; control media.

2. MEDIA 1: Concentrated and subsequently diluted media: DMEM-F12 (10X) or DMEM (10X) diluted with sterile water suitable for cell cultures and neutralised with a sodium bicarbonate solution at 7.5% to obtain a neutral pH. Afterwards it is supplemented with serum, glutamine and antibiotics; diluted control media.

3. MEDIA 2: Concentrated and subsequently diluted media: DMEM-F12 (10X) or DMEM (10X) diluted with sterile water suitable for cell cultures electrolysed at 33% of the maximum H_2 level, and neutralised with a sodium bicarbonate solution at 7.5% to obtain a neutral pH. Afterwards it is supplemented with serum, glutamine and antibiotics; diluted control media.

4. MEDIA 3: Concentrated and subsequently diluted media: DMEM-F12 (10X) or DMEM (10X) diluted with sterile water suitable for cell cultures electrolysed at 100% of the maximum H_2 level, and neutralised with a sodium bicarbonate solution at 7.5% to obtain a neutral pH. Afterwards it is supplemented with serum, glutamine and antibiotics; diluted control media.

Two treatments are carried out on cells: acute or chronic. The acute treatment is based in the addition of media with hydrogen water to subsequently carry out the cell viability assay according to the previously mentioned times. In chronic treatment, the media is changed daily until the assay is carried out.

All determinations are carried out in triplicate with 10 independent experiments.

After adding the cells with different media, they receive an acute or chronic treatment, and are left to incubate during the required time until carrying out the MTT assay, also known as Succinate Dehydrogenase Inhibition (SDI) assay.

The MTT assay is a colorimetric assay to assess the metabolic activity of cells in the active proliferation phase. It is based in the breaking of the tetrazolium ring of the MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Sigma-Aldrich, St. Louis, MO, USA), by different mitochondrial dehydrogenases, which form formazan crystals. The quantity of created formazan is proportional to the number of (metabolically active) proliferating cells.

A solution of 5mg/ml MTT in a phosphate buffer (PBS) is prepared, and 20µL for every 180 µL of fresh medium is added and incubated for 1 hour in HeLa cells or 2 hours in ARPE-19 cells. Afterwards, the medium is removed from the wells, it is washed with 100 µL of PBS and the resulting formazan crystals are solubilised in 200 µL of dimethylsulphoxide (DMSO). The culture plates are gently agitated for 30 minutes to break the cell membranes and solubilise the formazan. Finally, the optical density is measured at 550 nm using a microplate reader Synergy H1 Hybrid Multimode (BioTek Instruments, Winooski, VT, USA). The absorbance values are normalised in the controls.

Cell Antioxidant Activity assay (CAA)

ARPE-19 and HeLa cells are plated in microplates of 96 wells and incubated for 72h in an acute treatment. For the chronic treatment, cells are treated with Media 0 or Media 3 for 72h, by changing the media daily.

After three days the culture medium is removed and a pre-treatment is carried out. This pre-treatment consists in adding the hydrogenated PBD where necessary, Trolox ((±)6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid, a water-soluble synthetic equivalent of vitamin E) as an antioxidant pattern at concentrations ranging from 0 to 2000 µM. At the same time, the fluorescent probe of 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), which is permeable to cells, is incubated and retained inside the cells thanks to the action of intracellular esterases. To develop this assay, cells are maintained in PBS under the following conditions.

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Acute treatment:

1. PBS control.
2. Concentrate PBS and dilution with water.
3. Concentrate PBS and dilution with electrolysed water at 33% of the maximum H_2 level.
4. Concentrate PBS and dilution with electrolysed water at 100% of the maximum H_2 level.

Chronic treatment:

1. PBS control.
2. Concentrate PBS and dilution with water.
3. Concentrate PBS and dilution with electrolysed water at 100% of the maximum H_2 level.

This treatment is carried out during 60 min at 37°C. Afterwards, the cells are washed and incubated again with 200 µL of the corresponding PBS to which we subsequently add a free radical source: the 2,2’-Azobis(2-methylpropionamidine) (AAPH). Immediately after adding the AAPH, a fluorescence kinetics is started with a reading every 2 min during 1h by maintaining the plate at 37°C. The readings are carried out at an excitation wavelength of 485 nm and an emission wavelength of 525 nm.

The DCFH-DA quickly oxidises due to free radicals with 2',7'-dichlorodihydrofluorescein (DCF), which is highly fluorescent. The assay measures the capacity of hydrogen presence to prevent the formation of DCF due to peroxyl radicals, which are generated by AAPH in normal and tumour human cells. The reduction of cell fluorescence compared to control cells indicates the antioxidant capacity of compounds (Figure 1A). The results are expressed in CAA and in micromolar (µM) of Trolox equivalents (TE). Each plate includes tripled Trolox wells for each concentration and for blanks (contains a probe without oxidant). All other determinations are carried out in triplicate from five independent experiments. At the end of the reading time, cell viability is processed using the MTT method.

The Cell Antioxidant Activity is quantified using the normalised Area Under the Curve (AUC) with regard to the number of living cells (MTT) in each well. Finally, to process the data obtained along the assay, the results of each sample are interpolated with the Trolox pattern curve (CAA units vs [Trolox, µM] to obtain the Trolox Equivalents, using the computer programme GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) (Figure 1B).

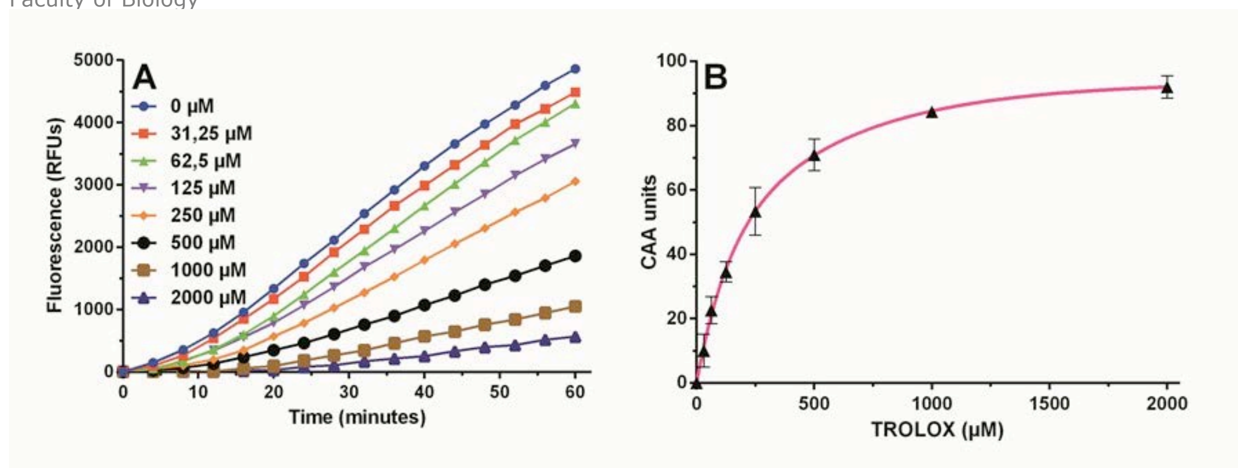

Figure 1. Plots of Trolox Kinetic Curves. (A) Representative fluorescence curves from CAA assay of varying concentrations of Trolox antioxidant standards ranging from 0 to 2000 µM. **(B) Dose-Response Curve of Trolox Standard.** The net AUC of varying concentrations of Trolox antioxidant standards ranging from 0 to 2000 µM were used to calculate CAA units that are plotted versus Trolox concentration. The subsequent calibration curve was used to interpolate the Trolox Equivalents (TE) value of the active compounds.

Results

Viability studies and cell proliferation

ARPE-19 and HeLa cells were cultured under optimum conditions and monitoring was carried out using optic microscopy to assess its evolution and the adequacy to carry out the experiments.

To assess the effect of hydrogen water on cell viability, the cells were incubated (72 h) with H_2 in the acute treated culture medium (an initial addition) or the chronic one (daily change of the medium with H_2); and the cell viability was assessed using the MTT assay. Figure 2A shows the results of the ARPE-19 cell line. These results indicate a normal clonal growth of cells in the control medium. The presence of hydrogen in the single addition of the culture medium (acute treatment) or the repetitive addition (chronic treatment) does not either modify the viability or the cell growth. These results confirm the safety of hydrogen water on healthy cell growth.

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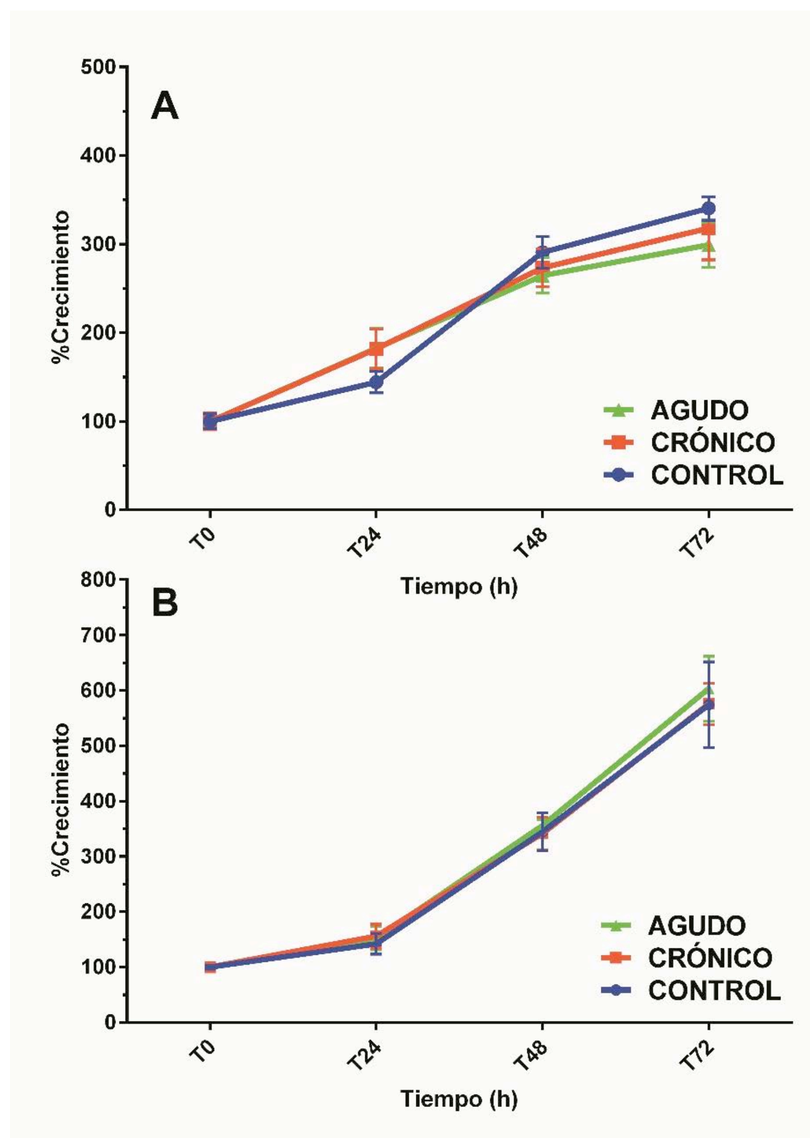

Figure 2. Time curves for electrochemically reduced water effect on the viability of the ARPE-19 (A) and HeLa cells (B). Cells were treated with cell culture medium with (Acute and Chronic treatment) or without H_2 (Control) and allowed to grow for 72 h. Chronic treatment involves changing the culture medium daily. Viability was measured by the MTT assay.

A similar study has been carried out with HeLa tumour cells (Figure 2B). Firstly, it should be pointed out, as expected, that the HeLa cells show a higher cell growth with regard to the ARPE-19 cells, as a consequence of its greater clonal growth. With regard to the H_2 effect in the culture medium, like in ARPE-19 cells, no alteration of the viability or the cell proliferation is

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observed. This result confirms the absence of cytotoxicity of hydrogen water, independently of the clonal cell proliferation degree.

Cell Antioxidant Activity (CAA)

To determine the production of intracellular free radicals, cells were treated with non-polar and non-fluorescent 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). The compound is subject to a deacetylation by cytosolic esterases to form the polar and non-fluorescent dichlorodihydrofluorescein (DCFH), which produces the fluorescent derivative dichlorofluorescein after reacting with free radicals (ROS).

The ARPE-19 and HeLa cells were cultured as a single layer in a microplate of 96 wells. Cells were pre-treated with DCFH-DA in PBS with and without H_2. After 1 hour of incubation, the medium was removed and the cells were washed with PBS. As a benchmark antioxidant, different concentrations of Trolox incubated in PBS with DCFH-DA and without H_2 were used at the same time. Then, the free radical source AAPH was added to cells to start the assay and the fluorescence was read every two minutes during 1 hour at 37°C. The fluorescence kinetics associated to the DCF formation was measured during 1h. The reduction of the Cell Antioxidant Activity (CAA) is defined in the percentage of intracellular free radicals with regard to the control without antioxidants and is quantified using the Area Under the Curve (AUC). It can also be expressed as the Trolox Equivalents concentration (TE) (Figure 1).

Table 1. Effect of the dose and the treatment in the Cell Antioxidant Activity (CAA) of hydrogen water in healthy and tumour cells

	ARPE-19		HELA	
	ACUTE	CHRONIC	ACUTE	CHRONIC
CONTROL	6.28 ± 2.66	10.50 ± 2.30	12.73 ± 2.01	16.18 ± 2.16
TREATED (33% H_2)	33.71 ± 6.91	N.D.	39.45 ± 8.04	N.D.
TREATED (100% H_2)	41.84 ± 2.55	62.37 ± 6.50	52.32 ± 7.54	45.85 ± 4.28

The antioxidant activity and the protective effect of hydrogen water were confirmed in ARPE-19 and HeLa cells which were exposed to the specific peroxyl radicals AAPH inducer (Table 1). The basal CAA is greater than the HeLa tumour cells compared to ARPE-19 cells due to its higher basal metabolism, which implies the need of a greater intracellular protection. In both cell lines, the acute addition of H_2 to the medium increases the CAA in a dose-dependent manner, with a higher increase of HeLa cells compared to ARPE cells. Although HeLa cells have a greater CAA at

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a quantitative level, a relative analysis compared to the basal analysis suggests a higher increase in ARPE-19 cells (>6,7) compared to HeLa cells (>4,1).

The average life of H_2 is of at least one hour, since no significant changes are observed in the protection kinetics during this time. The translation of the CAA into TE shows the same behaviour (Table 2). The antioxidant protection of hydrogen water is equivalent to the intake of a Trolox concentration of 260 µM in ARPE-19 cells and of 430 µM in HeLa cells, respectively.

Table 2. Effect of the dose and the treatment in the Cell Antioxidant Activity (CAA), expressed as Trolox Equivalents (TE), of hydrogen water in healthy and tumour cells

	ARPE-19		HELA	
	ACUTE	CHRONIC	ACUTE	CHRONIC
CONTROL	15.07 ± 4.64	35.83 ± 4.80	36.73 ± 7.98	50.48 ± 3.85
TREATED (33% H_2)	163.98 ± 30.19	N.D.	226.92 ± 42.36	N.D.
TREATED (100% H_2)	258.14 ± 46.80	669.12 ± 96.56	432.25 ± 61.35	317.41 ± 52.72

In a chronic treatment which simulates the daily intake of hydrogen water, the two cell lines show different behaviours. At a basal level, the daily change of the culture medium does not significantly modify the cell CAA in any of the two systems (Table 1). The presence of H_2 in this medium triggers a greater increase in the CAA of ARPE-19 cells with regard to acute treatment. This behaviour is not observed in HeLa cells where the chronic treatment of a medium with H_2 does not increase the CAA. The translation into TE shows the same behaviour with an equivalence of 670 µM of Trolox in ARPE-19 cells and of 320 µM in HeLa cells, respectively (Table 2).

This different behaviour of chronic treatment compared to acute treatment may be explained with two reasons. Firstly it could be related to the fact that a chronic administration of H_2 may induce an increase of the cell antioxidant system both in enzyme systems (SOD, GR, GPx, Cat,…) and chemical systems (glutathione,...). The non-increase observed in tumour cells could be explained due to the proven absence of some basic components of the cell antioxidant system, such as GPx, which prevents this strengthening. Similar behaviours between healthy and tumour cells have already been described for other active principles.

A second alternative could be related to the recently described fact (*Hamasaki, T.; Harada, G.; Nakamichi, N.; Kabayama, S.; Teruya, K.; Fugetsu, B.; Gong, W.; Sakata, I.; Shirahata, S.*

Electrochemically reduced water exerts superior reactive oxygen species scavenging activity in HT1080 cells than the equivalent level of hydrogen-dissolved water. PLoS One 2017, 12, e0171192) which indicates that a part of the residual CAA of hydrogen water may be related to the accumulation of platinum nanoparticles with antioxidant activity. Although this study has used HT1080 tumour cells and we have not seen any additive effect in the chronic treatment of HeLa cells, we cannot exclude the fact that the observed increase in ARPE-19 cells in the chronic treatment may be related to the accumulation of these nanoparticles.

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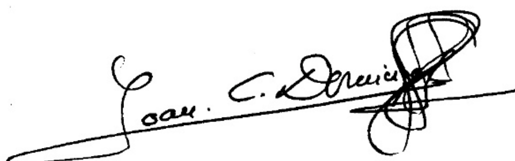

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