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Cellular toxicity induced by different pH levels on the R3230AC rat mammary tumour cell line. An in vitro model for investigation of the tumour destructive properties of electrochemical treatment of tumours

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Abstract

Introduction: The aim of this study was to evaluate the cellular toxicity of different pH levels on the R3230AC mammary tumour cell line (clone-D) in vitro and to determine in what way the pH affects the tumour cells. The results could be used to interpret the cell damaging effects seen in electrochemical treatment of tumours (EChT), where pH alteration in tissue is the major event. *Methods*: Tumour cells were treated with pH 3.5, 5, 7, 9, 10 and 11 for 10, 20 or 30 min, respectively, followed by studies with the viability assay 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (methyltetrazolium (MTT)), morphological observation in phase contrast microscope (PCM) and light microscope, nucleotide analogue incorporation (BrdU; 5-Brdmo-2′-deoxyuridine), Caspase-3 activity measurement and detection of DNA fragmentation by an agarose gel electrophoresis. *Results*: In the viability assay, it was found that different pH levels had cytotoxic effects; these effects were dependent on the pH value and on the time of exposure at a given pH. Morphologically, cells in pH 3.5 and 5 had shrunk, were rounded and had condensed chromatin, whereas prominent cell swelling and nuclear expansion were seen in the pH 9- and 10-treated cells. Gross cytolysis was found in pH 11. A BrdU incorporation assay indicated that proliferation rate is inhibited markedly both with decreasing and increasing pH. Significant Caspase-3 activity was found in pH 3.5 and 5 groups. Caspase-3 levels for the alkaline exposure were equal or below the normal control. DNA ladder formation, a characteristic of apoptosis, was only visualised in the treatment of pH 3.5 for 30 min. *Conclusions*: pH changes inhibit cell proliferation and decrease cell viability. The pathway of killing tumour cell in low pH probably has at least two directions: apoptosis and cell necrosis, whereas high pH results in only cell necrosis. The study suggests that low pH environment can induce apoptosis in unphysiological condition comparable with tissue pH at EChT. In addition, it seems that R3230AC mammary tumour cells are more tolerant to high pH than to acidic changes. This supports the theory that anodic EChT should be more efficient than cathodic.

Keywords: R3230AC; Electrochemical treatment; Caspase-3

1. Introduction

Electrochemical treatment of tumours (EChT) is a promising method and complement in modern oncology by which direct current is delivered into neoplastic tissue to induce tumour regression. EChT has been suggested as an effective local therapy [1–6]. Recent studies, both in vivo and in vitro, have confirmed its effectiveness in animal tumour models [7,8]. Human experience, primarily from China, showed that EChT inhibits proliferation of tumours [9].

The development of EChT as a clinically accepted therapy has somewhat been hindered by uncertainties regarding the mechanism of tumour destruction. Several different hypothesis of the destruction mechanism behind EChT have been proposed [1,10]. The main reaction products at electrolysis of biological tissue with insoluble electrode material are at the anode, which causes decomposition of water into molecular oxygen and hydrogen ions, and oxidation of chloride ions. While at the cathode, the electrolysis causes disintegration of

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water, which produces molecular hydrogen and hydroxyl ions, thereby, the tissue becomes acidified or alkalized, respectively. Platinum is the most commonly used electrode material in EChT. The effect of pH alteration in tumour tissue is probably the most important mechanism. Tumour destruction caused by EChT can be correlated with an unphysiological change in pH. Specific pH values known to cause total tissue destruction could be used as markers to evaluate the efficacy of treatment [11].

Even though EChT has been applied in clinical studies of different kinds of tumours and has shown positive response, there has been almost no systematic research in this field and the biological mechanisms are also poorly understood. Few studies have been carried out where the influence of pH on tumour cells has been analysed. It is not well known if the pH gradient, by itself, can induce apoptosis or cell death and to what extent the pH affects tumour cells and inhibits proliferation. A significant pH gradient by EChT can produce a fiercely cytotoxic local environment.

In this in vitro model, R3230AC mammary tumour cells (clone-D) have been incubated with specific pH values for 10, 20 or 30 min. After this, a ''postexposure recovery'' for 2–6 h was provided. The following methods for detecting cellular damage were applied: viability assay 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT), morphological observation in phase contrast microscope (PCM) and light microscope, nucleotide analogue incorporation (BrdU; 5-Bromo-2′-deoxyuridine), Caspase-3 activity measurement and detection of DNA fragmentation by agarose gel electrophoresis.

The aim of this study was to evaluate the cellular toxicity of different pH levels on the R3230AC mammary tumour cell line (clone-D) in vitro and to determine in what way the pH affects the tumour cells regarding proliferation and cell death. The results could be used to interpret the cell damaging effects seen in electrochemical treatment (EChT) of tumours, where pH alteration in tissue is the major event. With this model, we have focused on the effect of the pH on cellular damage in vitro, since the situation is considerably more complex in vivo. In this way, the mechanisms of tumour regression with EChT successively can be understood.

2. Materials and methods

2.1. Cell culture

The R3220AC rat mammary tumour cell line (clone-D; kindly provided by Prof. G. Bussolati, University of Turin, Italy) was cultured in RPMI 1640 with Glutamax I medium (GIBCO™, Invitrogen, Stockholm, Sweden) supplemented with 10% heat-inactivated foetal bovine serum in a humidified 37 °C, 5% CO_2 incubator. When BrdU and different pH were used, cells were cultured in either 2 ml of culture medium in plastic tissue culture dishes, 2 cm in diameter (Falcon, Becton Dickinson, NJ, USA) for morphological analysis (PCM or light microscopy) or in 10 ml of culture medium in plastic tissue culture dishes with 10 cm diameter for the other experimental assays.

2.2. General study design

The cells were studied in the following pH: 3.5, 5, 7, 9, 10 and 11. The different pH levels were achieved by adding sodium hydroxide or hydrochloric acid to culture medium. Durations used were 10, 20 and 30 min for all different pH-levels. To assure that the pH did not change during the incubation, pH measurements were preformed immediately after completing the CO_2 incubation. After the pH exposure, the cells were allowed to recover in culture medium with normal pH before studies were performed. The recovery periods were 2 h (methyltetrazolium (MTT) and Caspase-3), 3 h (BrdU and morphology) and 6 h for the DNA-ladder study.

2.3. Cell morphology

To determine alteration in cellular morphology, cultured cells attached to the plate were exposed to different pH and duration. After recovery for 3 h in culture medium with normal pH, observations were made under a phase contrast microscope, and subsequently, the cells were fixed in 4% formaldehyde, dried and stained using Giemsa. Further observations were made under a light microscope.

2.4. Viability test

Cell viability was measured using the MTT assay (Sigma, St. Louis, MO, USA). The 3-(4, 5 dimenthylthiazol-2-yl)-2,5,-tetrazolium bromide MTT is converted to an insoluble formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. In living cells, active mitochondrial dehydrogenase will cause this conversion, dead cells will not [12]. Cells were cultured in a 96-well culture plate (Nunc, Roskilde, Denmark), and about 4000 cells in 100 μl/well were added and incubated for 48 h at 37 °C. This was followed by a change of the normal medium to a pH-adjusted as described above. After pH treatment, the culture media was changed back to normal and the incubation continued for 2 h. Then MTT stock solution (5 mg/ml) was added to equal one tenth of the original culture medium, according to the manufacturer's protocol. The incubation was continued for another 4 h. At the end of the incubation period, adding acidic isopropanol solubilized the converted dye. The absorbance was measured at a wavelength of 570 nm on a microplate spectrophotometer (Molecular Devices, USA).

2.5. Cell proliferation

To study the effects of pH on cancer cell proliferation, cells were grown for 3 h in different pH conditions and assessed for BrdU incorporation.

The culture medium was changed and BrdU labelling solution (final concentration 10 μM in culture medium) was added and incubated for 3 h at 37 °C. The dishes were then washed twice with 2 ml washing buffer (phosphate-buffered saline (PBS) pH 7.4 with 10% FBS; foetal bovine serum). After this, the dishes were dried at 60 °C for 1 h and subsequently chilled in −20 °C for 2 min. The cells were fixed with 2 ml 4% formaldehyde for 30 min. Finally, they were rinsed with tap water for 20 min and left upside-down to dry overnight. BrdU incorporation into proliferating cells was determined immunohistochemically.

After incubation at 37 °C with 1 M HCl for 30 min to break down the hydrogen bonds and separate DNA, the dishes were washed with tris-buffered saline (TBS) for 2 × 5 min. To block endogenous peroxidase activity, 0.7% H_2O_2 solved in methanol were added for 30 min in RT. The dishes were rinsed with tap water for 2 min, with TBS for 5 min and treated with trypsin (1 ml 0.1% trypsin in TBS) for 5 min in 37 °C to improve antibody penetration, followed by careful rinsing with TBS. Blocking buffer (1 ml of 5% bovine serum albumin (BSA)–TBS) was applied for 15 min, followed by the incubation with the primary antibody (mouse anti-BrdU, Dako, Glostrup, Denmark) for 1 h in RT. After carefully washing, the secondary antibody (rabbit antimouse biotinylated, Dako) was applied and incubated for 30 min. The Avidine–Biotin Complex (Dako) was added for 30 min and DAB (diaminobenzidine, Sigma) for 7 min. Then counterstaining with Giemsa was performed according to standard procedures. The labelling index (LI), the ratio of BrdU positive cells and the total amount of cells were quantified by light microscopy. About 1000 cells were counted.

2.6. Caspase-3 activity

Caspase-3 activity was measured using the CPP32/Caspase-3 Fluorometric Protease Assay Kit (MBL, Naka-ku Nagoya, Japan) that recognises the sequence DEVD, based on detection of cleavage of substrate DEVD-AFC (AFC: 7-amino-4-trifluoromethyl coumarin). Cells (1×10^6) were cultured in 2 ml plastic dishes and pH-treated as described in the general study design. After incubation for 2 h in culture medium with normal pH, cells were scraped off and transferred to a 1.5-ml eppendorf tube, centrifuged and resuspended in 50 μl of chilled Cell Lysis Buffer and incubated on ice for 10 min. Then 50 μl of two times Reaction buffer (containing 10 mM dithiotreitol, DTT) and 5 μl of 1 mM DEVD-AFC Substrate (50 μM final concentration) were added to each sample and the cells were incubated in 37 °C for 1 h. Samples were transferred to a 96-well plate for reading in a fluorometer with 400 nm as excitation wavelengths and 505 nm emission wavelengths.

2.7. DNA electrophoresis

DNA electrophoresis was performed using a slightly modified method with a DNA extracting Kit (Sigma, Mammalian Genomic DNA kit G1N-70). Briefly, after the pH incubation and a 6-h recovery period, the attached cells were washed with PBS and harvested via a scraper and transferred to a test tube with PBS. $2–5 \times 10^6$ cells were centrifuged at $1200 \times g$ for 5 min. After the supernatant has been removed, the pellets were resuspended thoroughly in a 200-μl resuspension solution, subsequently treated with RNase 20 μl (20 mg/ml) and 20 μl proteinase K (20 mg/m) and lysed with 200 μl volume of lysis buffer at 70 °C for 10 min. Then 200 μl ethanol (95%) was added, and the sample was transferred to a preassembled GenElute nucleic acid binding column and centrifuged at 6500 rpm for 1 min. Eluted DNA was precipitated according to standard procedures overnight and resuspended in 15 μl of TE (tris-EDTA; ethylene-diamine-tetra-acetic-acid) buffer (pH 7.4). Loading buffer was added to the samples, and electrophoresis was performed in a 1.5% agarose gel for 180–240 min at 65 V. The DNA was visualised under UV-light with ethidium bromide.

2.8. Statistical analyses

Numerical results were expressed in figures as the mean with standard deviations (SD). Student's paired *t*-test was employed to compare data from the control and different pH exposure for each pH point. Values of $p < 0.05$ were considered indicative of statistical significance.

3. Results

3.1. Cell morphology

Morphological changes were observed under phase contrast and light microscopes. Cells treated with pH 7 showed normal morphology. In contrast, most of the pH 3.5- and 5-treated cells had shrunk, condensed chromatin, nuclear fragmentation, lost cell-to-cell contact and in part had detached from the plate. In the pH 9- and 10-treated cells, additional cell swelling (cytosolic oedema) and nuclear expansion were found. Cytoplasmic vacuolisation increased as the pH became more alkaline. Gross cytolysis was a distinctive feature for the cells in pH 11. The cell morphology changes were more prominent as the duration of exposure increased, especially in pH 3.5 and 5. But all the features were present already after 10-min exposure (Fig. 1A–E). Many of these characteristics suggested apoptotic morphology.

3.2. MTT viability assay

Cell viability was measured using the MTT assay. Mitochondrial respiratory function was significantly impaired by pH adjustment in a pH-dependent and time-dependent manner (Fig. 2). The effects were most prominent by pH 3.5, 10 and 11 for 30 min ($p < 0.01$).

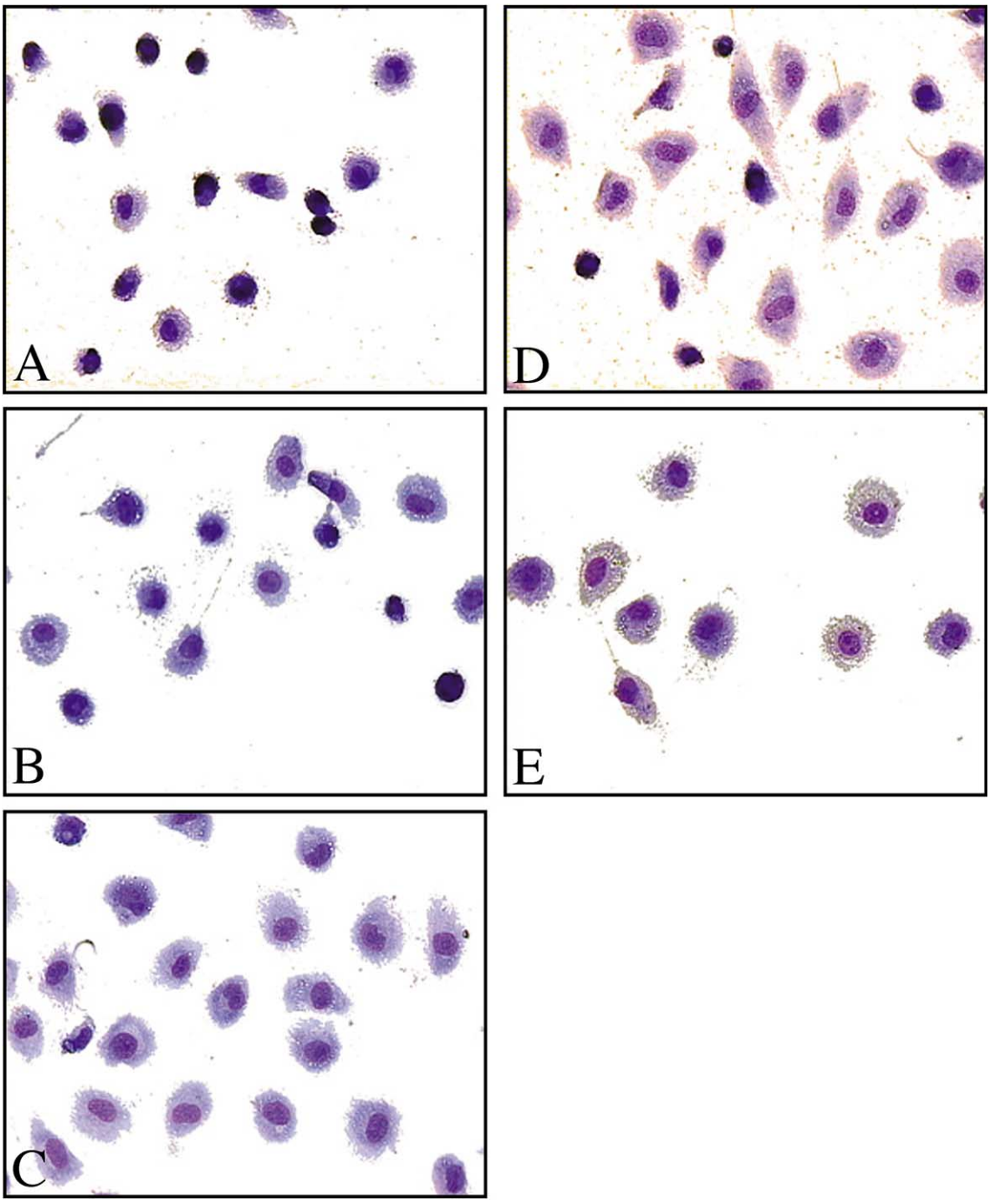

Fig. 1. R3220AC rat mammary tumour cell line (clone-D) treated with different pH for 10 min, fixated in formalin and stained with Giemsa, Magnification 200 ×. (A) pH 3.5, the cells are easily detaching from the plate surface. Remaining cells are shrunken, have pycnotic nucleus and condensed chromatin. (B) pH 5, as for (A), except that the number of cells has increased and pycnosis is less prominent. (C) pH 7, normal control. (D) pH 9, a mixture of normal and necrotic cells. Some cells have a swollen cytoplasm. (E) pH 11, few cells remaining. All cells have a rounded and swollen cytoplasm. The cells show an intracellular oedema, nucleus swelling and some have even disrupted plasma membranes.

3.3. BrdU labelling

Three consecutive experiments were performed. The number of cells counted in each plate was about 1000, except for the pH 3.5 and pH 11 for 30 min, where intact cells were absent. In pH 3.5 for 20 min and pH 11 (10 and 20 min), only one out of the three experiments contained perceptible cells. Hence, no standard deviations were stated. The result of the BrdU assay is shown in Fig. 3. In pH 7, the BrdU incorporation, labelling index (LI), varied between 64.8% and 93.9% (mean 80.5%), irrespectively of the duration of ‘‘exposure’’. Significantly decreased BrdU in-

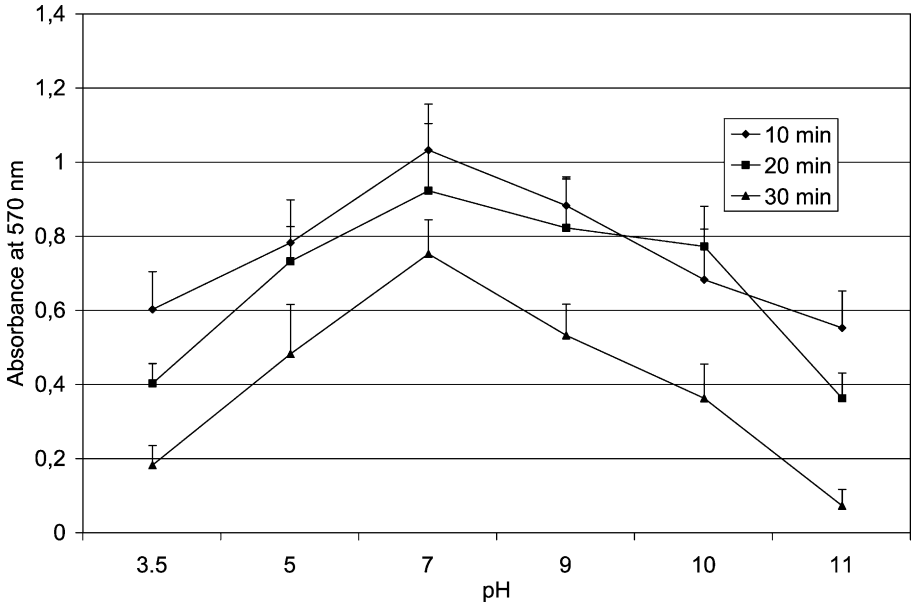

Fig. 2. Cell viability measured using the MTT assay. Each pH-point is made up of three independent experiments. Results are displayed as the mean absorbance at 570 nm with standard deviations (SD). There is a marked decrease in cell viability as the pH_e increases or decreases, respectively. The most significant changes ($p < 0.01$) were detected for pH 3.5, 10 and 11 for 30-min exposure.

corporation (paired *t*-test, $p < 0.05$) compared to normal was found in pH 3.5 (10 and 20 min), pH 9 (30 min), pH 10 (30 min) and pH 11 (10 and 20 min).

3.4. Caspase-3 activity

Significant rise in the Caspase-3 activity was found in all acidic treatment groups ($p < 0.05$), except for pH 5 for 20 min, still Caspase-3 activity was 54% higher than normal in this group (Fig. 4). The fold-increase in CPP32 activity was determined by comparing the results with the negative control, pH 7 (value 1). No significant differences between normal control and the alkaline group could be detected.

3.5. Detection of DNA fragmentation by agarose gel electrophoresis

Treatment of the cells with pH 3.5 for 30 min created a pattern characteristic of internucleosomal fragmentation,

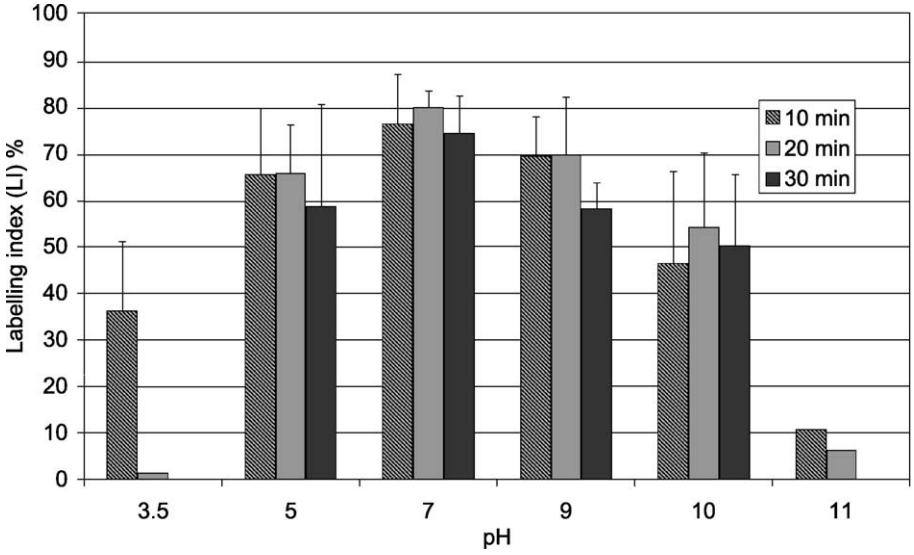

Fig. 3. Measurement of the thymidine analogue, BrdU, incorporation or labelling index (LI). BrdU is a specific S-phase marker. The labelling index (LI), BrdU positive cells divided with the total cell number, was quantified in 10 light microscopic high-power fields randomly selected. Normal labelling index varied between 64.8% and 93.9% (mean 80.5%). Significantly decreased BrdU incorporation (paired *t*-test, $p < 0.05$) compared to normal were found. Error bars show the standard deviation (SD).

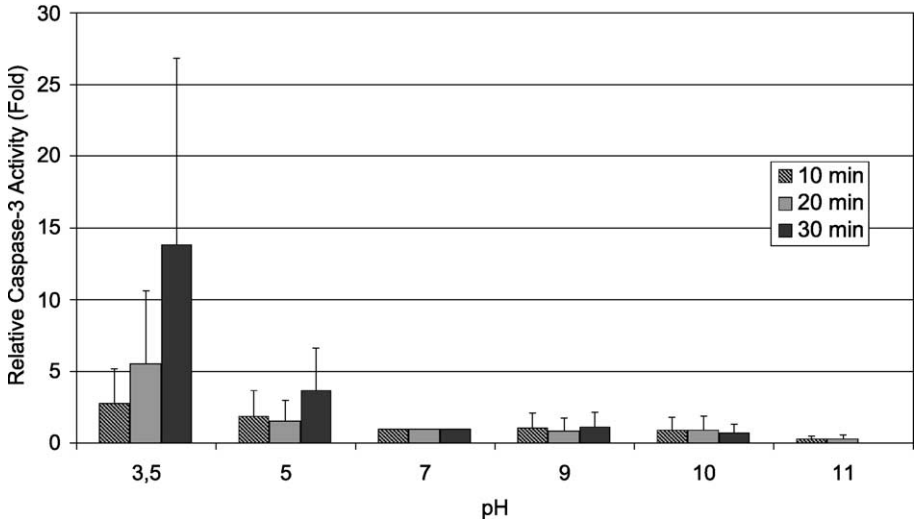

Fig. 4. Measurement of the Caspase-3 activity. Significant rise in the Caspase-3 activity, an early detector of apoptosis, was found in all acidic treatment groups ($p < 0.05$) except for pH 5 and 20 min, still Caspase-3 activity were 54% higher than normal in this group. No significant differences between normal control and the alkaline group could be detected. The CPP32 activity is described as fold-increase with standard deviations (SD), compared to negative control pH 7 (value 1).

DNA ladder (Fig. 5). Treatment of cells with other pH and duration of exposure did not cause marked DNA fragmentation. Agarose gel displayed high molecular weight genomic DNA that remained trapped in or near the well, while smaller fragments down to 180 base pairs were resolved.

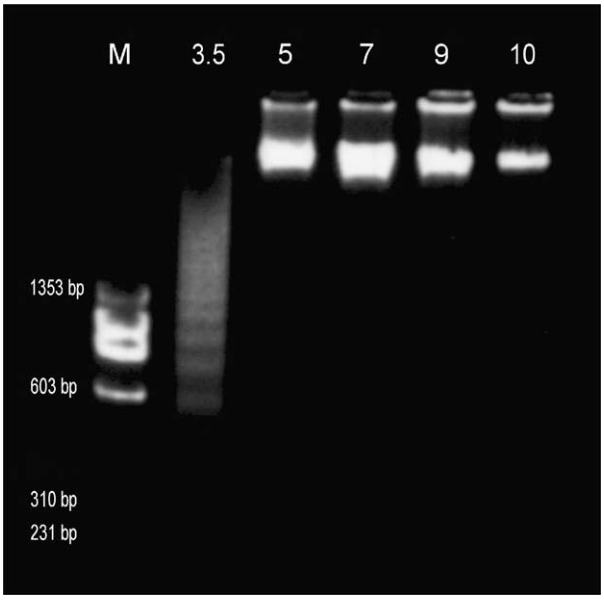

Fig. 5. Detection of DNA fragmentation by agarose gel electrophoresis (1.5% agarose gel for 240 min at 65 V). pH values are marked above each lane, exposure time: 30 min. M = DNA fragment size marker. Treatment of the cells with pH 3.5 and 30 min created a pattern characteristic of internucleosomal fragmentation, DNA ladder.

4. Discussion

Ever since it was first introduced in Sweden in the early 1980s, EChT has gained its main clinical interest in China [9]. Recent reports have shown that EChT is more considered as one of the alternatives for local tumour destruction, even in the Western world [13,14]. Both earlier and recent reports claim that the pH alterations in tissue during electrolysis are the main reason for the success with electrochemical treatment [1,10,11]. However, there are few data concerning the effects of electrochemical treatment regarding the biological and biochemical changes that are altered, which could further explain the success with this treatment. Hence, this mono factor experiment, in vitro, has a unique ability to reveal what actually occurs when tumour cells are exposed to acidic and alkaline stress, respectively, at levels comparable to what is seen in EChT.

The buffer capacity of the body has an important impact on the ability of the tissue to react on electrolysis in vivo. Several reports have been published where the buffer systems have been analysed both theoretically and experimentally [15–17]. In this study, the cells were allowed to recover from toxic insult due to variable pH for 2–6 h before samples were analysed regarding viability, proliferation, apoptosis, etc. The addition of normal medium can mimic the organism's engagement of the different buffering systems during and immediately post-treatment with EChT. In this way, the persisting cell injuries after pH treatment are displayed.

The alteration of the pH value has important biological consequences to cancer cells. It does not only modify the cytotoxicity of some anticancer drugs, but also induces cell damage or even cell death [18].

Phase-contrast and light microscopy revealed an increase in the number of cells that had lost cell-to-cell contact and had detached from the plate with increased, as well as decreased, pH and longer periods of incubation. Most of the detached cells, in low pH, showed apoptotic morphology with secondary degeneration in Giemsa stain (Fig. 1).

Inspection in light microscope of the pH-treated cells expressed the same characteristics as found in EChT [1,4,19]. The cells in the acidic group had pycnotic nuclei, condensed chromatin, shrunken in size, and for the pH 3.5 for 30 min group, many of the cells had detached from the plate. In the alkaline group, the cells were swollen, had loose chromatin, and in the highest pH, the plasma membrane had disrupted.

In EChT, a strong acidification and dehydration take place around the anode, which lead to the cellular features as described above. At the cathode, the prominent alkalization and cellular oedema result in chromatin and plasma membrane characteristics as seen with the R3230AC mammary tumour cell line treated with high pH in vitro. The dehydration or oedema respectively is due to the migration of water as it acts like a dipole, electroosmosis, whereas the pH changes are referable to the hydrolysis of water [15]. The cytopathology were more mixed in the alkaline group, where shorter exposure (10 and 20 min) to pH 9 did not change the cellular morphology significantly compared to the normal control. This suggests that alkaline stress is less toxic than acidic stress.

Substantial changes in the extracellular pH induced a significant decrease in cell viability judged by methyltetrazolium (MTT) metabolism, which decreased significantly compared to the control in all modalities except for pH 9 (10 and 20 min, respectively). The results suggested that both exposure time and pH level are correlated to the destructive effect, and that low pH is more aggressive in creating a toxic environment.

The BrdU is incorporated into DNA as a structural analogue of thymidine. It serves as a specific S-phase marker [20]. Proliferation of tumour can be assayed with BrdU immunocytochemistry. In this study, the tumour cells dose-dependent, time-dependent growth inhibition is shown in Fig. 3. To inhibit the cell proliferation, it seems important to maintain an unphysiologic pH for a certain time. However, it is not clear how acidification and alkalization affect the BrdU incorporation. The changes of pH to levels of below 4 and above 10 affect cancer cells dramatically. We can expect that the intracellular pH (pH_i) and ionic concentrations change as the extracellular pH (pH_e) does and then induce cell damage or even death [21]. Hence, pH alteration and its effects on cancer cells is probably an important mechanism of EChT.

It is reported that exposure of cells to an acidic environment or intracellular acidification by inhibiting the pH_i regulatory mechanisms causes apoptosis [22,23]. Acidification might be a signal for DNA digestion. It is conceivable that the activity of the enzymatic reactions, as well as other multistep cellular and biochemical processes are greatly influenced by environmental acidity. We used the R3220AC rat mammary tumour cell line (clone-D) because it is very stable and works well even in an in vivo milieu allowing tumour inoculation studies to be performed.

Several authors claim that acidification of the intracellular environment is an essential condition for the induction of apoptosis, and that DNAse II with an optimal pH of about 5.0 is responsible for the DNA fragmentation in apoptosis [24,25]. In the present study, we clearly demonstrated that at low pH values, apoptosis is enhanced. Acidic environment in pH 3.5 resulted in the degradation of DNA and a ladder formed of oligonucleosome-sized fragments, a characteristic of apoptosis. DNA electrophoresis demonstrated that pH-induced apoptosis were both dependent on the pH-level, as well as the duration of exposure. To detect inevitable changes in the DNA towards apoptosis, the Caspase-3 activity measurement served as a suitable complement. With this, early events in the apoptotic process, not seen with morphological or electrophoresis experiments and not requiring the presence of intact cells, can be detected and quantified. Cysteine proteases (e.g. Caspase-3) are present in most mammalian cells and are unusual in a way that they cleave proteins after aspartate residues and they themselves require activation by cleavage. This suggests that a cascade mechanism of activation may exist. However, the target proteins of these caspases have not been fully identified. When the R3220AC rat mammary tumour cell line was exposed to different pH, it clearly reacted with apoptotic degeneration in all treatment modalities with acid media. We could therefore conclude that the increase of apoptosis in an acidic medium, as observed in the present study, results from intracellular acidification.

In higher pH, most cells in this study were markedly necrotic. DNA electrophoresis demonstrated high molecular weight smear bands; DNA ladder formation did not occur (Fig. 5). No early apoptotic degradation could be detected even with the Caspase-3 assay (Fig. 4). This suggests that cell injury in alkaline conditions is not due to apoptotic degeneration. The mechanisms are preferably that higher alkalization condition directly destroys various enzyme and structure protein, and result in inactivation of function or protein denaturation. Intracellular alkalization increases the inward calcium current and results in an increase of intracellular Ca^{2+} [26]. The initiating event in necrosis leads to an influx of water and extracellular ions. Intracellular organelles, most notably the mitochondria, and eventually the entire cell, swell and rupture, the structure of the DNA is randomly degraded. Acute cellular oedema is the most characteristic feature of tissue treated with cathodic EChT.

The extracellular pH change inhibits proliferation and decreases viability. The pathway of killing tumour cells in low pH probably has at least two directions: apoptosis and cell necrosis (mainly apoptosis), whereas high pH results in cell necrosis. This implicates that EChT, with its ability to

create a fiercely toxic pH gradient, is an effective way of treating tumours.

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References

[1] B.E. Nordenström, Biologically Closed Electric Circuits: Clinical, Experimental and Theoretical Evidence for an Additional Circulatory System, Nordic Medical Publications, Stockholm, 1983.
[2] E. Azavedo, G. Svane, B. Nordenstrom, Radiological evidence of response to electrochemical treatment of breast cancer, Clin. Radiol. 43 (1991) 84–87.
[3] G. Sersa, D. Miklavcic, U. Batista, S. Novakovic, F. Bobanovic, L. Vodovnik, Anti-tumor effect of electrotherapy alone or in combination with interleukin-2 in mice with sarcoma and melanoma tumors, Anticancer Drugs 3 (1992) 253–260.
[4] D.T. Griffin, N.J. Dodd, J.V. Moore, B.R. Pullan, T.V. Taylor, The effects of low-level direct current therapy on a preclinical mammary carcinoma: tumour regression and systemic biochemical sequelae, Br. J. Cancer 69 (1994) 875–878.
[5] Y. Matsushima, E. Takahashi, K. Hagiwara, C. Konaka, H. Miura, H. Kato, Y. Koshiishi, Clinical and experimental studies of anti-tumoural effects of electrochemical therapy (ECT) alone or in combination with chemotherapy, Eur. J. Surg. Suppl. 574 (1994) 59–67.
[6] Y.L. Xin, The clinical advance in application of EChT within the past ten years, Preprints from the 2nd International Symposium on Electrochemical Treatment of Cancer, 27–30 Sep., Beijing, China, 1998, pp. 81–92.
[7] S.A. Wemyss-Holden, G.S. Robertson, P.M. Hall, A.R. Dennison, G.J. Maddern, Electrolytic treatment of colorectal liver tumour deposits in a rat model: a technique with potential for patients with unresectable liver tumours, Dig. Dis. 18 (2000) 50–57.
[8] Y. Yen, J.-R. Li, B.-S. Zhou, F. Rojas, J. Yu, C.K. Chou, Electrochemical treatment of human KB cells in vitro, Bioelectromagnetics 20 (1999) 34–41.
[9] E. Nilsson, H. von Euler, J. Berendson, A. Thörne, P. Wersäll, I. Näslund, A.-S. Lagerstedt, K. Narfström, J.M. Olsson, Review—electrochemical treatment of tumours, Bioelectrochemistry 51 (2000) 1–11.
[10] D. Miklavcic, G. Sersa, M. Kryzanowski, S. Novakovic, F. Bobanovic, R. Golouh, L. Vodovnik, Tumor treatment by direct electric current—tumor temperature and pH, electrode material and configuration, Bioelectrochem. Bioenerg. 30 (1993) 209–220.
[11] H. von Euler, E. Nilsson, A.-S. Lagerstedt, J.M. Olsson, Development of a dose-planning method for electrochemical treatment of tumors. A study of mammary tissue in healthy female CD-rats, Electro- Magnetobiol. 18 (1999) 93–105.
[12] C.G. Tepper, S. Jayadev, B. Liu, A. Bielawska, R. Wolff, S. Yonehara, Y.A. Hannun, M.F. Seldin, Role for ceramide as an endogenous mediator of Fas-induced cytotoxicity, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8443–8447.
[13] S.A. Wemyss-Holden, G.S. Robertson, A.R. Dennison, P.M. Hall, J.C. Fothergill, B. Jones, G.J. Maddern, Electrochemical lesions in the rat liver support its potential for treatment of liver tumors, J. Surg. Res. 93 (2000) 55–62.
[14] S.A. Wemyss-Holden, P.M. Hall, G.S. Robertson, A.R. Dennison, P.S. Vanderzon, G.J. Maddern, The safety of electrolytically induced hepatic necrosis in a pig model, Aust. N.Z. J. Surg. 70 (2000) 607–612.
[15] J. Berendson, J.M. Olsson, Bioelectrochemical aspects of the treatment of tissue with direct current, Electro- Magnetobiol. 17 (1998) 1–16.
[16] E. Nilsson, J. Berendson, E. Fontes, Development of a dosage method for electrochemical treatment of tumours: a simplified mathematical model, Bioelectrochem. Bioenerg. 47 (1998) 11–18.
[17] H. von Euler, E. Nilsson, J.M. Olsson, A.-S. Lagerstedt, Electrochemical treatment (EChT) effects in rat mammary and liver tissue. In vivo optimizing of a dose-planning model for EChT of tumours, Bioelectrochemistry 54 (2001) 117–124.
[18] I.J. Furlong, R. Ascaso, A. Lopez Rivas, M.K. Collins, Intracellular acidification induces apoptosis by stimulating ICE-like protease activity, J. Cell Sci. 110 (1997) 653–661.
[19] K. Li, Y. Xin, Y. Gu, B. Xu, D. Fan, B. Ni, Effects of direct current on dog liver: possible mechanisms for tumor electrochemical treatment, Bioelectromagnetics 18 (1997) 2–7.
[20] H.G. Gratzner, Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: a new reagent for detection of DNA replication, Science 218 (1982) 474–475.
[21] P.A. Stewart, Modern quantitative acid–base chemistry, Can. J. Physiol. Pharm. 61 (1983) 1444–1461.
[22] H.J. Park, C.M. Makepeace, J.C. Lyons, C.W. Song, Effect of intracellular acidity and ionomycin on apoptosis in HL-60 cells, Eur. J. Cancer 32 (1996) 540–546.
[23] H.J. Park, J.C. Lyons, T. Ohtsubo, C.W. Song, Acidic environment causes apoptosis by increasing caspase activity, Br. J. Cancer 80 (1999) 1892–1897.
[24] M.A. Barry, J.E. Reynolds, A. Eastman, Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification, Cancer Res. 53 (1993) 2349–2357.
[25] J. Li, A. Eastman, Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na(+)/H(+)-antiport, J. Biol. Chem. 270 (1995) 3203–3211.
[26] R.D. Smith, D.A. Eisner, S. Wray, The effects of changing intracellular pH on calcium and potassium currents in smooth muscle cells from the guinea-pig ureter, Pflugers Arch. 435 (1998) 518–522.