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Comparison of cytotoxic changes in ECV304 cell through exposure to DMSO and sodium butyrate using impedance spectroscopy

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Abstract

This paper decides an impedance spectroscopy measurement system for real-time monitoring of cellular toxicity. The system is used to compare the impedance response of ECV304 cells to two toxicants, dimethyl sulphoxide (DMSO) and sodium butyrate. The responses at different concentration of toxicants were plotted in 3-D impedance spectra and their correlation coefficients were calculated at four time points. Additionally, Phase contrast microscopy images were used to observe the effect of the two toxicants on the morphology change of the cells. The characteristic information of the spectra compared well with the morphological changes of cells, illustrating that the method is useful for monitoring cytotoxicity effects and cell viability.

Keywords: Impedance spectroscopy; toxicant;

1. Introduction

Cytotoxicity measured using impedance spectroscopy is a non-invasive, label-free analytical method. Impedance measurement using electrodes has become an important tool to determine changes in cell physiology, migration and proliferation in living cells. The impedance value which differs with cell types is a measure of the transfer function, relating the output voltage to the input current. Impedance measurement systems have been used in cell biology for several decades. Electric cell-substrate impedance sensing system [1] is based on using a small micro-electrode with a large counter electrode to measure cellular impedance. It has been demonstrated as a successful method to detect cellular activity in vitro toxicology [2,3,4]. In addition, Cho and Becker [5] designed a microelectrode-based chip in the study of virus-induced effects in Vero cells at low virus concentrations. They concluded that the

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impedance parameters relating to the cell/cell or the cell/substrate junction were more sensitive to diagnose the virus infection than microscopic observations of cell detachment. Wang et al. [6] designed a real-time, label-free impedance system to monitor the progression of the cell cycle. They concluded that the time-course impedance curves mirror the progression of the cell cycle clearly, and the shapes of these curves are closely connected with a degree of synchronicity of the cell population. Yeon et al. [7] used a micro-fabricated cell chip to monitor cell cytotxicity of human hepatocellular carcinoma cells after treatment with several toxicants. Diemert et al. [8] used an impedance-based system to detect kinetics of cell death in different neuronal cell lines. Their results showed that the changes of impedance depended to a great extents on the severity of the insult. In this study, an impedance spectroscopy measurement system is used to plot the impedance spectra for cellular response to two toxicants. The correlation coefficients can distinguish the difference of toxin activity. The system enables measurement of cellular response in a noninvasive and label-free manner.

2. Materials and measurement system

ECV304 cells were seeded in a 75 cm^3 ventilated plastic well and maintained in complement 199 medium to achieve full differentiation. Cells were routinely replenished with fresh medium every 2 days and were inspected daily under the light microscope to check for confluence until a monolayer of adherent cells covers 75-80% of the surface of the well, where cells were seeded at the same concentration of 2×10^5 cells/well. When confluence was reached, the cell layer was first rinsed twice with 5ml Hanks Balanced Solution. They were then treated with 1ml of 0.25% Trypsin EDTA solution to cover the surface of the enzymatic digestion of cell layer. The well was finally placed in the incubator and left for approximately 5 minutes to allow the cell layer to detach. The trypsin was stopped with 5 ml of complete 99 Medium. ECV304 cells were collected and split at ratio of 1:10. Cells were maintained at 37 C^0 in a humidified atmosphere containing 5% CO_2 atmosphere. DMSO and sodium butyrate were supplied from Sigma Aldrich (UK). They were prepared in four concentrations 0.5%,1.25%, 2.5% and 5%.

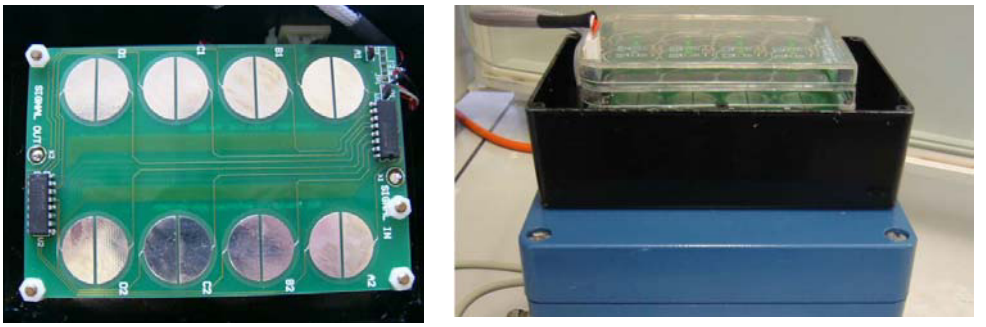

Fig.1 (a) Top view of impedance analyzer, which is connected to a PCB with an array of eight electrodes; (b) Front view of impedance analyzer, where a plastic well is placed on the PCB.

The measurement system consists of two parts: a multi sensor array and an impedance analyzer. The impedance analyzer is capable of processing eight wells independently. A D-shape sensor with a diameter of 22.1mm fabricated using PCB techniques was used, as shown in Fig.1. A plastic chamber was placed on the PCB sensor and the impedance analyzer was connected to a PC for data acquisition and processing. The time varying electrical signal is an ac signal and the frequency of the ac signal was varied

from 400 kHz to 2400 kHz. These frequencies are located in the beta dispersion region [9], where the external electric field can penetrate into the cell interior.

Phase contrast time-lapse microscopy was using for cell morphological monitoring. During the measurement, the chamber was placed on an inverted microscope. Image of cells were obtained by using a Nikon inverted contrast microscope with a digital camera.

3. Impedance spectra

The analysis was made on ECV304 cellular response during twenty hours exposure to each toxicant. A frequency range between 400 kHz to 2400 kHz was studied. The sampling frequency space between two individual frequency was 10 kHz. It took three minutes to cycle through the whole frequency range. Investigation in beta dispersion region is ideal to analyse cellular activity in a biological system that can be represented by an electrical model. Two kinds of the spectra were collected from the impedance analyzer; one representing the ECV304 cells response to a specific concentration of toxicant, and the second from ECV304 cells without toxicant, as a control. The output spectra in Fig.2-3 (a), (b),(c) and (d) were determined by the subtraction results from two spectra. It was shown in Fig.2 (a),(b) and (c), the majority of the peaks were located below 1200 kHz frequency. The spectra became stable after eight hours exposure to DMSO. Compare with results in Fig.2 (a), (b) and (c), the result in Fig.2 (d) were not tightly clustered. It was shown in Fig.3 that the majority of the peaks were located below 800 kHz frequency. The spectra became after three hours exposure to sodium butyrate.

The dissipation factor is defined as follows:

$$a = \frac{R}{X} \tag{1}$$

Where R is the resistance and X is the reactance. The resonance frequency is defined as the frequency at which the reactance is zero. Based on the Eq.1, the dissipation factor will reach a maximum at the resonance frequency.

Cross correlation is a method of estimating the degree to which two time series are correlated. Consider two time series A_i and B_i ,where $i = 0,1,2...N-1$. The cross correlation r is defined as:

$$r = \frac{\sum (A_i - \overline{A})(B_i - \overline{B})}{\sqrt{\sum (A_i^2 - \overline{A})^2} \sqrt{\sum (B_i^2 - \overline{B})^2}} \tag{2}$$

where $\overline{A}$ and $\overline{B}$ is the mean value of two series A_i and B_i .

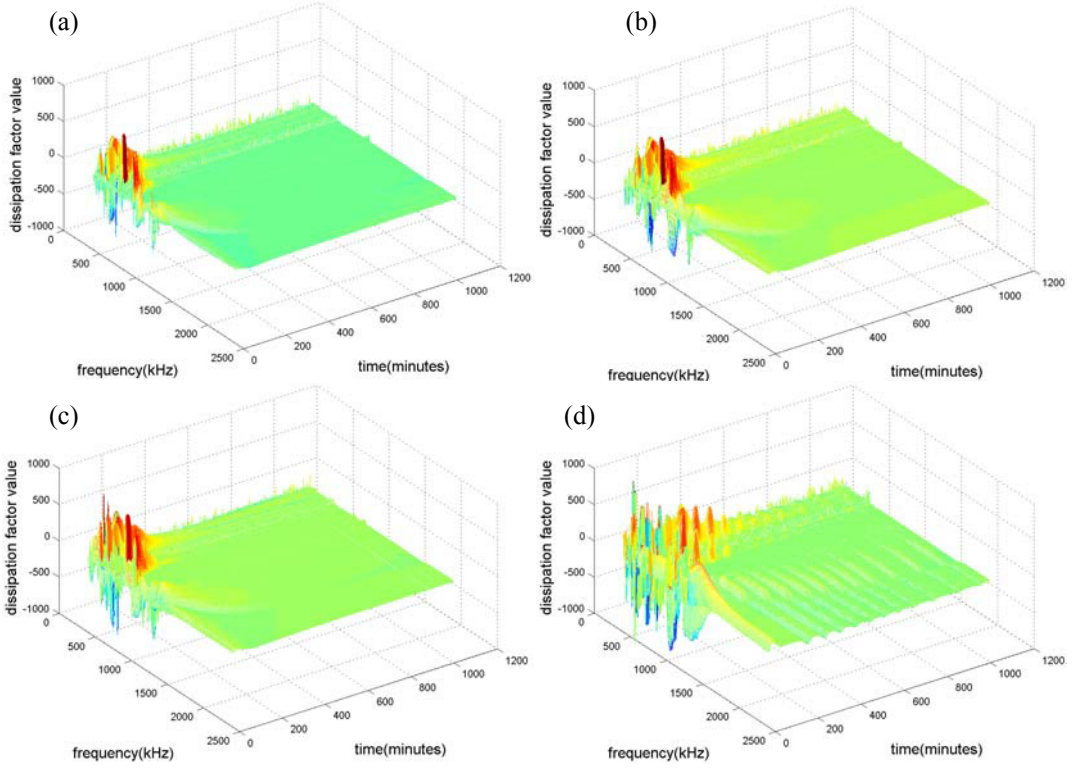

Fig.2 3D impedance spectra that represent the ECV304 cellular effects to DMSO at concentration (a) 0.5% DMSO (b) 1.25% DMSO (c) 2.5% DMSO (d) 5% DMSO.

The spectra in the range (800 kHz- 2400 kHz) at four time points are shown in Fig.4-5. The curves representing the acute toxicants such as 5% DMSO and 5% sodium butyrate were completely different with the other measured time points. It is shown in Fig.4 that clear peaks occur in the frequency range 800 kHz to 1000 kHz. When the concentration increased from 0.5% to 1.25% DMSO, the peaks in Fig.4 right shifted by 40 kHz. When the concentration increased from 1.25% to 2.5% DMSO, the peaks right shifted by 10 kHz. At frequencies higher than 1600 kHz, the dissipation factor value had no significant change and the value at 5% DMSO was 50 units larger than the value at the low concentration. In Fig.4 (a)(b), the values at 1.25%/2.5% DMSO were 25 units larger than the value at 0.5% DMSO, and these values appeared no difference in Fig.4 (c)(d). In order to improve the case of comparison, the correlation coefficients at four measured time points were calculated in Table 1. The spectra at 0.5% DMSO is poorly correlated with the spectra at 1.5%/2.5% DMSO at first and second hour, whereas the spectra at 1.5%/2.5% DMSO are similar with correlation coefficients larger than 0.8. The spectra at three concentration of DMSO all had close relationship with correlation coefficients larger than 0.6 at third and fourth hour.

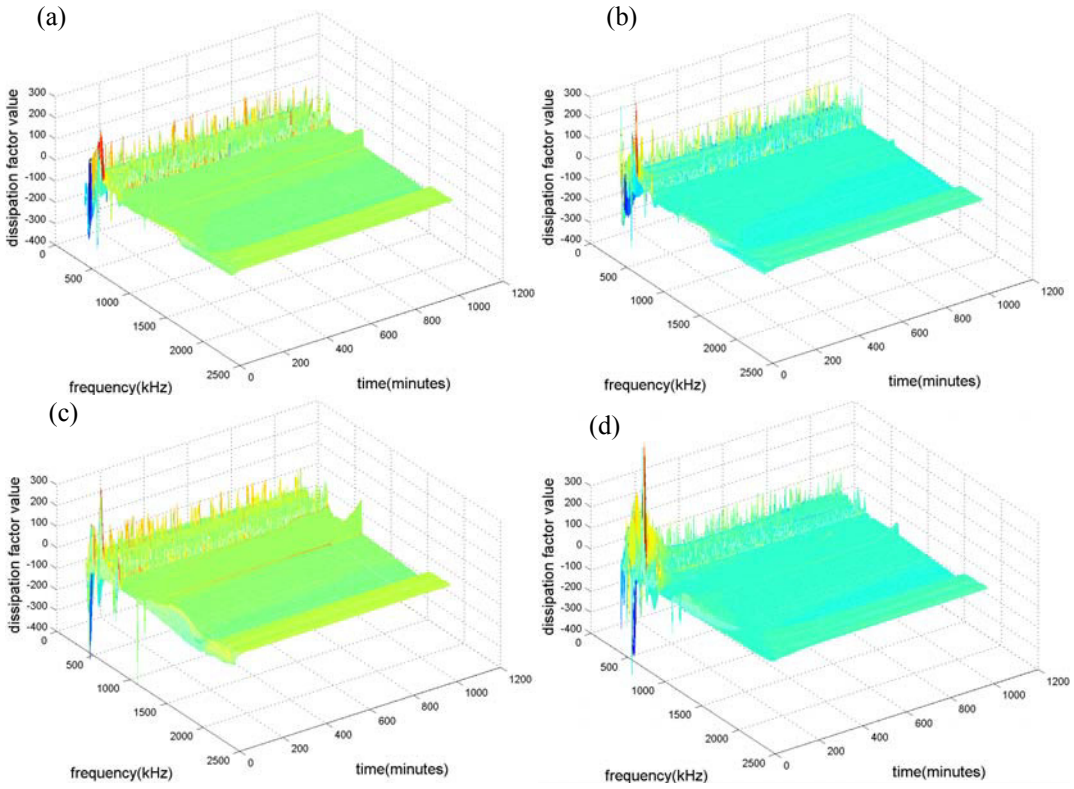

Fig.3 3D impedance spectra that represent the ECV304 cellular effects to Sodium butyrate at concentration. (a) 0.5% sodium butyrate; (b) 1.25% sodium butyrate; (c) 2.5% sodium butyrate; (d) 5% sodium butyrate.

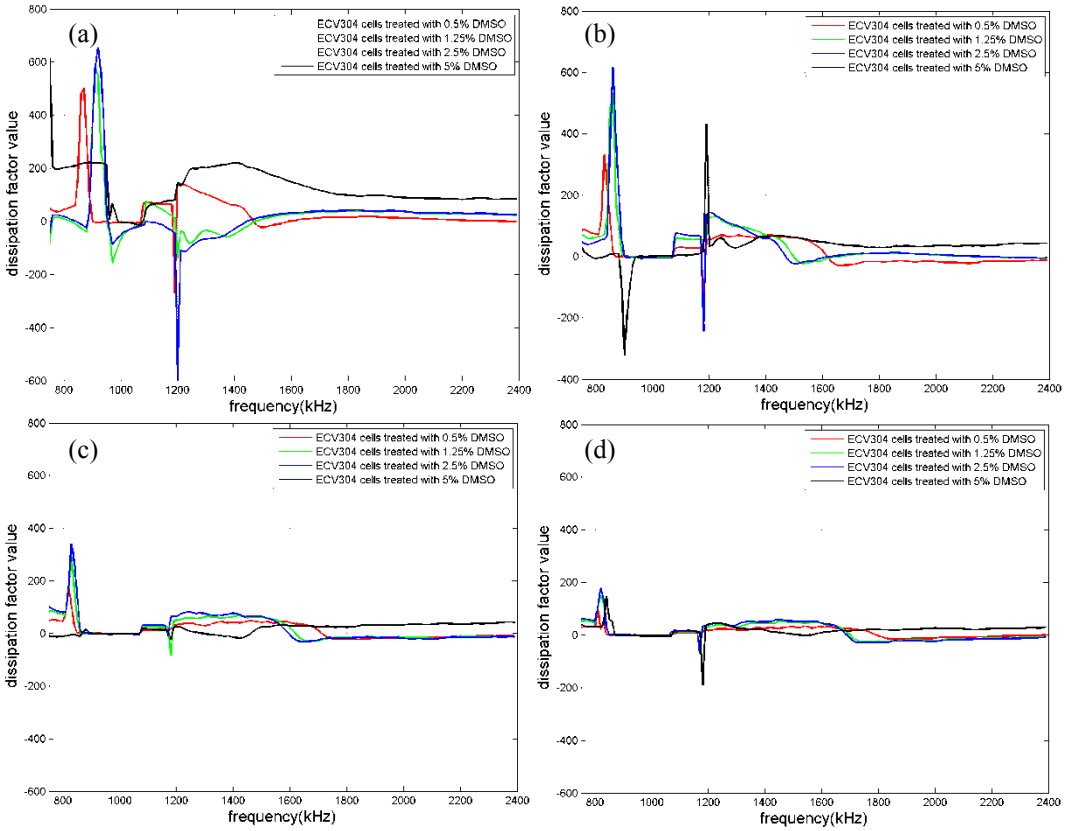

Fig.4 Impedance spectra of ECV304 cells treated with four different concentration of DMSO at different frequencies; (a) the spectra were measured at first hour; (b) the spectra was measured at second hour; (c) the spectra were measured at third hour; (d) the spectra were measured at fourth hour.

Cells treated with DMSO at 1st hour	0.5% DMSO	1.25% DMSO	2.5% DMSO
0.5% DMSO	1		
1.25% DMSO	0.1499	1	
2.5% DMSO	0.1906	0.8145	1
Cells treated with DMSO at 2nd hour	0.5% DMSO	1.25% DMSO	2.5% DMSO
0.5% DMSO	1		
1.25% DMSO	0.2997	1	
2.5% DMSO	0.1948	0.9039	1
Cells treated with DMSO at 3rd hour	0.5% DMSO	1.25% DMSO	2.5% DMSO
0.5% DMSO	1		
1.25% DMSO	0.7443	1	
2.5% DMSO	0.6052	0.9318	1
Cells treated with DMSO at 4th hour	0.5% DMSO	1.25% DMSO	2.5% DMSO
0.5% DMSO	1		
1.25% DMSO	0.8319	1	
2.5% DMSO	0.8369	0.9545	1

Table 1 Results of correlation coefficients for ECV304 cells treated with DMSO at different time.

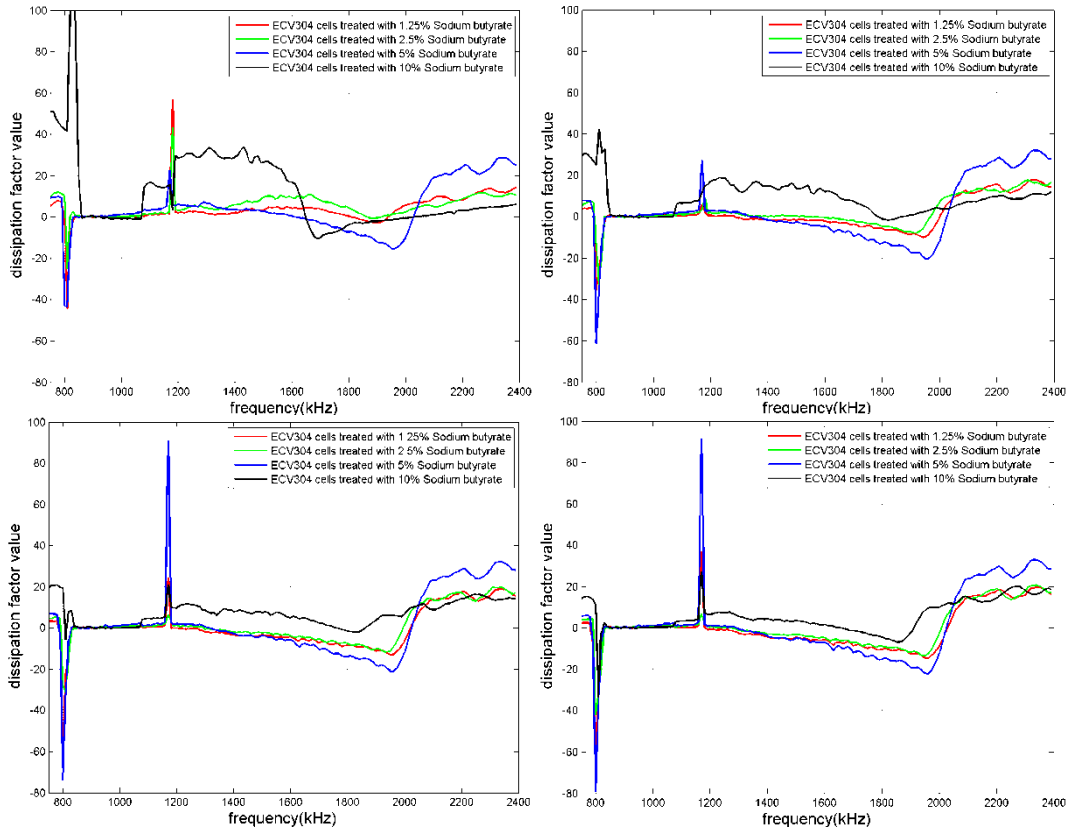

Fig.5 Impedance spectra of ECV304 cells treated with four different concentration of sodium butyrate at different frequencies. (a) the spectra were measured at first hour; (b) the spectra were measured at second hour; (c) the spectra were measured at third hour; (d) the spectra were measured at fourth hour.

Cells treated with Sodium butyrate at 1st hour	0.5% Sodium butyrate	1.25% Sodium butyrate	2.5% Sodium butyrate
0.5% Sodium butyrate	1		
1.25% Sodium butyrate	0.9243	1	
2.5% Sodium butyrate	0.3694	0.3964	1
Cells treated with Sodium butyrate at 2nd hour	0.5% Sodium butyrate	1.25% Sodium butyrate	2.5% Sodium butyrate
0.5% Sodium butyrate	1		
1.25% Sodium butyrate	0.6775	1	
2.5% Sodium butyrate	0.6179	0.3328	1
Cells treated with Sodium butyrate at 3rd hour	0.5% Sodium butyrate	1.25% Sodium butyrate	2.5% Sodium butyrate
0.5% Sodium butyrate	1		
1.25% Sodium butyrate	0.6024	1	
2.5% Sodium butyrate	0.6593	0.5927	1
Cells treated with Sodium butyrate at fourth hour	0.5% Sodium butyrate	1.25% Sodium butyrate	2.5% Sodium butyrate
0.5% Sodium butyrate	1		
1.25% Sodium butyrate	0.9210	1	
2.5% Sodium butyrate	0.7666	0.6282	1

Table 2 Results of correlation coefficients for ECV304 cells treated with sodium butyrate at different time

Fig.5 also shows the dissipation factor value as a function of frequency. There were peaks occurred in the frequency range 1000 kHz to 1200 kHz. However, compared with the results in Fig.4, these peaks did not shift when the concentration of sodium butyrate was increased. The relationships between the spectra representing ECV304 cells treated with the three concentration of sodium butyrate were determined by calculation of correlation coefficients in Table 2. The spectra at 2.5% sodium butyrate poorly correlated with the spectra at 0.5%/1.25% sodium butyrate at first and second hour, whereas the spectra at 0.5% and 1.25% sodium butyrate were similar with correlation coefficients larger than 0.6. the spectra all had close relationships with correlation coefficients larger than 0.6 at third and fourth hour.

It concluded that the toxic tolerance level of ECV304 cellular response to DMSO was between 2.5% to 5% DMSO, and the toxic tolerance level of ECV304 cellular response to sodium butyrate was between 2.5% to 5% sodium butyrate. If the concentration of the toxicant was controlled under the tolerance level, the results from the impedance analyzer would generate a unique curve pattern in the first two hours measurement.

4. Morphological changes

Fig. 6-7 show photo micrographs of ECV304 cells and the resulting morphological changes seen after 24 hours exposure to increasing concentrations of DMSO and sodium butyrate. The cells are allowed to grow to confluence creating a monolayer of cells that cover the base of the culture plate well. Fig.6 shows the changes observed when the cells are exposed to increasing concentrations of DMSO. It is thought that DMSO disrupts cytoplasmic membrane homeostasis by affecting transmembrane gradients of several physiological ions [10] resulting in osmotic damage and the induction of apoptosis [11]-[13]. Fig.6. E shows the monolayer of cells that were not exposed to DMSO which resembles cobblestones, called a cobblestone formation. All the cells are similar in size and no apoptotic cells are seen. As the concentration of DMSO is increased cellular changes become prominent. Fig.6 A-D show the progression of change for 0.5%, 1.25%, 2.5% and 5% respectively. A number of changes are seen, cells become swollen and enlarged cells are seen at 1.25% DMSO and all higher concentrations. At the highest concentration the cells were seen to be highly deformed and the monolayer formation was lost with large spaces between the cells evident. It can also be seen that the number of apoptotic cells increase as the dose of DMSO increases which are seen as “bright” round structures. With 0.5% DMSO the apoptotic structures are beginning to form, being smaller than those seen at higher concentrations. At 5% DMSO there are numerous, larger apoptotic bodies representing large number of dead cells. The photomicrographs show significant changes and damage to the cells at concentrations of DMSO higher than 1.25% DMSO.

Fig.7 shows photomicrograph pictures of ECV304 cells that were exposed to increasing concentrations of sodium butyrate. Previous studies report that sodium butyrate causes morphological changes, including increase in cell size [14][15] as a result of changes to the structure of chromatin (in the cell nucleus) and the cytoskeleton of the cell [16]. Fig. 7E shows the normal cobblestone appearance of a monolayer of cells which have not been exposed to sodium butyrate. The effect of increasing the dose of sodium butyrate, on cell morphology is seen in Fig. 7A -D, for 0.5%, 1.25%, 2.5% and 5% respectively. It can be seen that a small number of apoptotic structures are formed but the monolayer remains intact for all concentrations of sodium butyrate. The cell size is seen to be increased at 2.5 and 5% sodium butyrate, which is consistent with previous reports in the literature.

Over the concentration ranges of DMSO and sodium butyrate used to challenge ECV304 cells it can be seen that DMSO has most damaging effect on the cell morphology, and could be considered the most toxic. This is consistent with the finding shown in Fig.4-5. Impedance spectra for DMSO show significant similarity across all concentrations of DMSO. For 2.5% and 5% DMSO, they indicate little metabolic activity and a high degree of cell death, whereas the impedance spectra for sodium butyrate show differences between the different concentration of sodium butyrate, indicating different levels of cell activity and the cell are still alive.

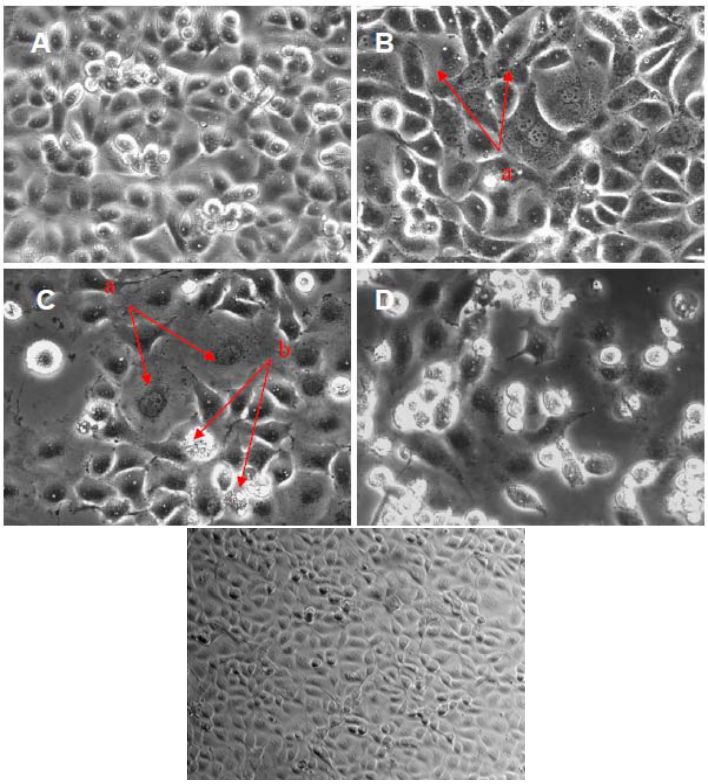

Fig.6 Morphological changes of ECV304 cells treated with various concentrations of DMSO during 24 hours. (A) 0.5% DMSO (B) 1.25% DMSO; (C) 2.5% DMSO; (D) 5% DMSO (E) Control cells.
Red arrows showed (a) large cells, (b) apoptotic structures

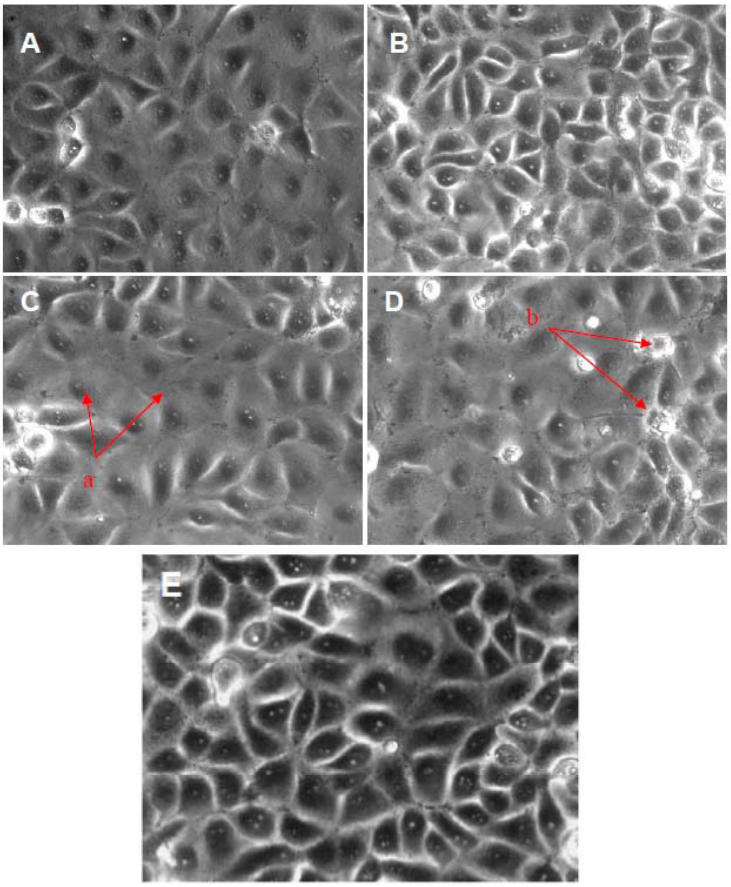

Fig.7 Morphological changes of ECV304 cells treated with various concentrations of Sodium butyrate during 24 hours. (A) 0.5% sodium butyrate (B) 1.25% sodium butyrate; (C) 2.5% sodium butyrate; (D) 5% sodium butyrate (E) control cells ;
Red arrows show (a) large cells, (b) apoptotic structures.

5. Conclusions

The impedance spectra associated with EVC304 cells have been monitored following exposure to two toxins: DMSO and sodium butyrate. The spectra were obtained over a frequency range of 800 to 2400 kHz and the dissipation factors were calculated from the impedance spectra hourly up to 4 hours after addition of the toxin. The impedance response to the toxin is concentration dependent. Furthermore, the spectra provide dynamic information about changes in the cells following the toxic challenge. Cross correlation of the spectra illustrated that the two toxicants differ in mode of action on the cells and that DMSO could be consider more toxic that sodium butyrate. Phase contrast microscopy reinforced these observations with DMSO having the most damaging effect on the cell morphology. In conclusion, cross correlation of has been shown to be an effective method to ascertain lack of cell viability and 3-D impedance spectra indicate different cytotoxicity effects.

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