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Electrochemical Sensing of Hepatocyte Viability

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Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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We investigated the use of amperometric and chronoamperometric methods with a double mediator system and screen-printed electrodes (SPEs) for the electrochemical sensing of hepatocyte viability. Cell counts were determined based on measuring cellular respiration via interaction of electroactive redox mediators. The oxidation currents of chronoamperometric measurement were proportional to the concentrations of ferrocyanide which produced via interaction of cellular respiration, succinate and ferricyanide. The integrated oxidation charges increased linearly with the density of the cultured primary rat hepatocytes over a range of 1×10^5 to 5×10^5 cells/well, (slope = 1.98(±0.08) μC/10^5 cells; R^2 = 0.9969), and the detection limit was 7600 (±300) cells/well based on S/N = 3. Each density of cells was cultured in triple replicates and individual cell samples were evaluated. The results of cytotoxic effect of the chronoamperometric method are comparable to those of the tetrazolium-based colorimetric assay. The chronoamperometric method with ferricyanide and succinate mediators is an efficient, alternative method for assessing the viability of primary hepatocytes. It can be completed in 20 min. Succinate did not provide an efficient electron shuttle between cytosolic respiratory redox activity of cancer cells and extracellular ferricyanide, an effect that may be useful for distinguishing heptacarcinoma cells from healthy hepatocytes.

1.Introduction

During the last 50 years, drug-induced liver injury has been the most frequent single cause of safety-related drug marketing withdrawals. Liver cell models for the evaluation of chemical hepatotoxicity play an increasingly crucial role in the development of new drugs.[1] Primary hepatocytes are a useful model for the analysis of the hepatotoxicity of new drugs. Spectrophotometry, fluorescent microscopy, or flow cytometry are used in the current methods of monitoring hepatocyte viability, and the use of each has advantages and disadvantages. Because fluorescent microscopy and flow cytometry instruments are expensive, spectrophotometric methods are most often used.

The colorimetric reagent most often used for assessing the viability or survival of cultured cells after drug treatment is 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT).[2, 3] Metabolism in viable cells produces NADH and NADPH, which reduce the MTT to a nonaqueous soluble formazan product, the quantity of which is directly proportional to cell viability. However, the MTT assay requires that cells be cultured in the presence of MTT for 3 to 4 h, before being solubilized with an organic solvent, such as Dimethyl sulfoxide (DMSO) or isopropyl alcohol. Resazurin is another commonly used reagent that allows the detection of viable cells based on the production of a red fluorescent product.[4] The resazurin-based assay can be completed in 10 min, but the reagent is expensive and unstable. Thus, improvements in the timeliness and cost-effectiveness of methods for detecting cell viability are needed.

Electrochemical measurements of the purine metabolic intermediates [5] or respiratory chain activity are used to evaluate cell viability in studies of chemical cytotoxicity. Direct or mediated electrochemistry based on a single- or double-mediator system has been used to study the redox activity of bacteria,[6, 7] fungi,[8-13] and cancer cells, including hepatoblastoma (HepG2),[14, 15] HL-60,[16] and MDA-MB-231[17] cancer cell lines. Electrochemical detection methods have not been used to assess the viability of isolated, healthy liver cells. However, Pemberton et al.[15] quantified cell number and monitored glucose metabolism in HepG2 cells by using an electrochemical method. *Escherichia coli* and other Gram-negative microorganisms can reduce ferricyanide directly, but mediators can improve electrochemical detection by providing an electron shuttle between intracellular reducing centers and an external electrode.[12] Zhao et al.[12] studied the use of

lipophilic mediators, including 2-methyl-1, 4-naphthalenedione (menadione) and 2,6-dichlorophenolindophenol (DCPIP), for assessing the redox activity of yeast, and determined that menadione interacts with the anaerobic respiration pathway, whereas DCPIP interacts with fermentation pathway. The compounds, menadione and DCPIP, form free radicals in redox reactions that are thought to be toxic to most cells.[18]

Cell membranes regulate the movement of chemicals in and out of the cell by using proteins embedded in the phospholipid bilayer and mediators that vary between different types of cells. [12] Both metabolism and the cell membrane structure differ between healthy cells and cancerous cells of a given cell type. The membrane glycoproteins of cancer cells are often overexpressed,[19] and metabolism in most types of cancer cells relies primarily on glycolysis,[20] leaving their mitochondria essentially uninvolved in ATP production. Thus, the electron flow in healthy liver cells may differ from that of cancerous cells.[17]

We examined the feasibility of the electrochemical assessment of hepatocyte viability by applying amperometric and chronoamperometric methods using a double-mediator system that consisted of lipophilic and hydrophilic mediators. The lipophilic mediator provided an electron shuttle between the intracellular reducing centers and the external hydrophilic mediators. Fig. 1 shows a representation of the reaction mechanism of the double-mediator system. The lipophilic mediator diffuses inside the cells, and is reduced by NADH and NADPH. The reduced lipophilic mediator then diffuses across the cell membrane into the extracellular matrix, and reacts with ferricyanide, recovering the oxidized form of the lipophilic mediator to complete the cycle.

Fig 1 here

Fig. 1. Mechanism of the electrochemical assessment of hepatocyte viability by means of a hydrophilic/hydrophobic redox indicator couple

The optimal concentrations of the electrochemical mediators were determined, and the results of the electrochemical assessments were compared with those of an MTT assay. Lipopolysaccharide (LPS) acts as a prototypical endotoxin because it binds the CD14 receptor complex, promoting the secretion of pro-inflammatory cytokines in several cell types, which may cause hepatocytotoxicity.[21, 22] Thus, we used LPS as a model drug to evaluate the feasibility of our electrochemical method to assess cell viability and quantify hepatocytotoxicity.

2. Materials and methods

2.1 Reagents and apparatus

Phosphate buffered saline (PBS), potassium ferricyanide $K_3[Fe(CN)_6]$, potassium ferrocyanide $K_4[Fe(CN)_6]$, DMSO, MTT, LPS, menadione, collagenase type IV, ethylenediaminetetraacetic acid (EDTA) and sodium succinate dibasic hexahydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). William E medium, Mg^{2+} and Ca^{2+} free Hank's balance salt solution (HBSS), the antibiotic mixture (50 μg ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, and 10 μg ml^{-1} neomycin), high-glucose supplemented Dulbecco modified eagle medium (DMEM), glutamine, and non-essential amino acids were purchased from Life Technologies (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit Haemek, Israel). Percoll®, a medium for density gradient centrifugation of cells, was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Culture dishes and microplates were obtained from Nunc (Roskilde, Denmark).

Electrochemical measurements were performed using screen-printed electrodes (SPE), each consisting of a carbon working electrode (3mm in diameter), a carbon counter electrode (meniscus shape with 1mm width), and a pseudo-silver reference electrode (1 mm in diameter). The electrodes were purchased from Zensor R&D (Taichung, Taiwan). The electrochemical measurements were conducted using a potentiostat (EmStat, Leicester, UK), and data acquisition was performed using the PSTrace software (PALM instruments BV, Houten, Netherlands). A FlexStation3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used for measuring absorbance for the MTT assay.

2.2 Cell culture

Primary hepatocytes were isolated from male Wistar rats (body weight, 200-250 g), which were obtained from BioLASCO Taiwan Co. The animal experiments in our study were approved by the Institutional Animal Care and Use Committee at Chung Shan Medical University (approval no. 890). The hepatocytes were prepared using the two-step collagenase perfusion technique, which involves sequentially perfusing the liver with EDTA and collagenase.[23] After anesthesia was induced, rat liver was perfused with HBSS containing EDTA for 15 min at 37 °C, and was subsequently perfused with 0.5 mg ml^{-1} of collagenase in HBSS for 15 min. Dead cells were removed by performing centrifugation 3 times at 500 rpm in Percoll. Rat hepatocytes were cultured in William E medium supplemented with 10% FBS, 2 mM of glutamine, and 1% antibiotic mixture at 37 °C in a humidified atmosphere with 5% CO_2. Rat primary hepatocytes were seeded in 24-well culture plates or in 10-cm culture dishes. Each density of cells was cultured in triple replicates and individual cell samples were evaluated. Cells were allowed to attach for 12 h before the cultured medium was replaced with fresh culture medium with (test samples) or without (controls) the cytotoxic testing chemicals. The HepG2 and Chang liver cells were subcultured from cell lines originally obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The HepG2 and Chang liver cells were cultured in DMEM in the same conditions as those used for the rat primary hepatocytes.

2.3 Cell viability assessments using the MTT assay

The medium was withdrawn from the cultured cells, and the cells were washed with PBS. The cells were incubated in fresh medium supplemented with MTT (0.5 mg ml^{-1}) at 37 °C for 4 h. After removing the MTT medium, DMSO was added to dissolve the formazan crystals, and the absorbance of formazan was measured at 560 nm. The relative viability was calculated according to the following equation, in which A_{blank} was the absorbance of the medium only; $A_{control}$ was the absorbance of the cells cultured without the testing chemicals (control sample), and A_{test} was the absorbance of the cells cultured with the testing chemicals (test sample):

$$\text{viability}\,\% = \frac{A_{test} - A_{blank}}{A_{control} - A_{blank}} \times 100\%$$

2.4 Cell viability assessments using the electrochemical method

The screen-printing carbon electrodes were electrochemically pretreated before use (see supplemental material Fig. S1). The medium was withdrawn from the cultured cells, and the cells were incubated for 20 min in a fresh medium supplemented with the electrochemical mediators. A 50-μL aliquot of the medium was applied to the surface of the SPE, and the oxidation current of the ferrocyanide was measured. The relative viability was calculated according to the following equation, in which i_{blank} was the oxidation current of the medium only; i_{control} was the oxidation current of the medium from the cells cultured without the testing chemicals (control sample), and i_{test} was the oxidation current of the medium of the cells cultured with the testing chemicals (test sample):

$$\text{viability}\,\% = \frac{i_{test} - i_{blank}}{i_{control} - i_{blank}} \times 100\%$$

2.5 Statistical analysis

All experiments were performed in triplicate. The data are expressed as the mean and standard deviation. The results of the chronoamperometric and MTT assays were compared using an analysis of variance (ANOVA) or a paired Student *t* test. A $P < 0.05$ was considered to indicate a statistically significant difference. The statistical analyses were performed using the Microsoft Excel computer program (Redmond, WA, USA).

3. Results and discussion

3.1 Optimization of electrochemical detection

We investigated the use of the lipophilic mediators, menadione and succinate, which are the most commonly used electrochemical mediators in the literature. The MTT assay was used to examine the effects of the lipophilic mediators on cell viability, and chronoamperometry was used to examine the electron shuttle effect. Fig.2a shows the effects of various concentrations of succinate and menadione on the viability of rat primary hepatocytes measured using the MTT assay. Both succinate and menadione reduced primary hepatocyte viability.

Fig.2b shows the oxidation current of ferrocyanide, which represented cell respiration, in the cells incubated with ferricyanide and various concentrations of menadione or succinate. The oxidizing potential was set at +0.4 V (*vs*. pseudo -Ag reference electrode) as previously described.[17] (see Fig S2 and S3) Although menadione allowed the electrons to be shuttled between the cell respiratory chain and the ferricyanide, high concentrations of menadione caused severe cytotoxicity, substantially reducing the electrochemical signal. High concentrations of succinate caused cytotoxicity to a lesser extent than that caused by menadione. Treatment with greater than 5 mM of succinate generated significant oxidation signals, indicating that succinate provided an electron shuttle between the cell respiratory chain and the ferricyanide.

Fig 2 here

Fig. 2. Effects of lipophilic mediators on cell viability and electron transport. (a) The viability of rat primary hepatocytes after incubating with medium supplemented with 5 mM ferricyanide and various concentration of lipophilic mediators. The viability was measured using the MTT assay. (b) The oxidation currents of ferrocyanide formed in the cell respiratory. (□ succinate, ■ menadione).

Fig. 3 shows the reaction time and concentration effects of the hydrophilic mediator, ferricyanide, on the sensitivity of the electrochemical detection method. We assessed the hydrophilic mediator by using 5 mM of succinate and various concentrations of ferricyanide. The signal increased with increasing reaction time. The signal was similar at various concentrations of ferricyanide for reaction times less than 10 min. The signal limitation was cell activity under these conditions. The respiratory cycle of the cultured cells produced a significantly measurable amount of ferrocyanide when the cultured medium was supplemented with 5 mM of ferricyanide and 5 mM of succinate and the reaction time was equal or longer than 20 min. Thus, these conditions were used in subsequent experiments designed to determine signal sensitivity, and LPS toxicity.

Two methods of electrochemical detection were investigated. One method involved using amperometric detection coupled with flow injection (FIA), and the other involved using chronoamperometry. The FIA system was coupled with an injector fitted with a 20-μL PEEK loop and a flow cell. A 3-electrode system was used for detection. The working electrode was a screen-printed carbon electrode, and the reference electrode was an Ag/AgCl electrode in saturated AgCl and a 3-M KCl solution. The counter electrode was

stainless steel tubing. The carrier was PBS (pH 7.4), and the flow rate was 0.6 mL min^{-1}. The 3-electrode-SPE used for the chronoamperometric measurements consisted of a carbon working electrode, a carbon counter electrode, and a pseudo-silver reference electrode. One drop of the sample was applied directly to the surface of the SPE electrodes, and the potential was set at +0.4 V.

Fig 3 here

Fig. 3. The reaction time and concentration effects of ferricyanide with 5 mM of succinate on the electrochemical detection. Rat primary hepatocytes ($3x10^6$ cells) were seeded in 10 cm culture dishes with 5 mL culture medium.

Fig. 4a shows that the linear range of the FIA system was 0.05-0.4 mM (y = 13.647x - 0.1034, R^2 = 0.9957), and the detection limit (S/N = 3) was 3.8 μM for ferrocyanide in PBS supplemented with 5.0 mM of ferricyanide and 5.0 mM of succinate. The current responses of ferrocyanide in William E medium supplemented with 10% FBS, 5.0 mM of ferricyanide, and 5.0 mM of succinate were indistinguishable from those of the blank solution, which may have resulted from proteins in the medium adsorbing to the carbon surface, blocking electron transfer and causing electrode fouling. The plot of the average limiting current at 39 to 40 s versus ferrocyanide concentration is shown in Fig. 4b. The slope of the calibration curve for ferrocyanide was 1.70 μA mM^{-1} (R^2 = 0.9961), and the detection limit (S/N = 3) was 8 μM. The use of a disposable SPE for each sample resolved the protein-adsorption and cross-contamination problems. No significant difference between ferrocyanide prepared in the PBS and in culture medium was observed. Therefore, the chronoamperometric method with disposable SPE was better for measurements of cell culture medium.

3.2 Cell counts assessed according to respiratory activity

The cell counts of rat primary hepatocytes, the HepG2 cancer cells, and the Chang liver cells were determined under the various assay conditions. The original data traces and example of data treatment were shown in supplemental material Fig.S4 and S5. When the potential was stepped to +0.4 V, the current flowing through an electrochemical cell included faradic (charge transfer) and nonfaradic current (capacitive current) at the early phase. The nonfaradic current did not involve any chemical reactions (charge transfer), it only caused accumulation (or removal) of electrical charges on the electrode and in the solution near the electrode and it faded rapidly to zero when the potential became constant again after potential step initiation. According to Cottrell equation, the faradic current of chronoamperometry is, on the other hand, proportional to $1/\sqrt{t}$ and as expected plots of oxidation currents versus $1/\sqrt{t}$ during the time periods of 5~45 s had a good linearity R2=0.9974)(see Fig S2b). This indicated that there was no interference of nonfaradic current at the chosen time period. Thus, charges used for the assessment of cell viability were integrated from 5 to 45 s using PSTrace software to prevent interference by capacitive currents. The plots of the integrated current responses (Q) versus the cell densities of the primary hepatocytes, the Chang liver cells, and the HepG2 cells are shown in Fig. 5. The ingredients of culture mediums were complicate and some of the components were electroactive, such as ascorbic acid. The culture mediums for primary hepatocytes, and the HepG2 cells were different. Therefore, the blank solution that contained culture medium, ferricyanide, and zero cells presented some background currents and varied for different culture mediums.

Fig 4 here

Fig. 4. Current response versus ferrocyanide concentration. (a) FIA with amperometric detection. (b) Chronoamperometric detection. The chronoamperometric current was the average of signals at time of 39~40 sec after application of +0.4 V vs. pseudo-Ag reference electrode. The ferrocyanide was prepared with 5.0 mM ferricyanide and 5.0 mM succinate in PBS(■) or in culture medium with 10% FBS(□)

The charges increased linearly (R^2 = 0.9969) over a cell density range of 1×10^5 to 5×10^5 cells/well for rat primary hepatocytes. Each density of cells was cultured in triple replicates and individual cell samples were evaluated. The reproducibility (well to well) of integrated responses was 0.4% for blank (culture medium without liver cells) and was 3% for cell densities of 1 $x10^5$ to $5x10^5$. The slope was 1.98 (±0.08) $\mu C/10^5$ cells, and the detection limit was 7600 (±300) cells/well based on S/N = 3. The HepG2 cell line is a liver hepatocellular carcinoma. The charges of the measurements of various HepG2 cell densities were analyzed using an ANOVA Statistical Analysis. The p value is 0.2884, which means there is no significant difference of the signals between blank and various cell densities. These results are consistent with those of our previous study,[17] which showed that succinate did not provide an efficient electron shuttle between cancer cells and ferricyanide. The slope of charges was only 0.54 (±0.04) $\mu C/10^5$ cells for the Chang liver cells. The Chang liver cell line was originally thought to be derived from healthy liver tissue, but the ATCC subsequently found them to be contaminated with HeLa cells. Therefore, the response was less than that of primary rat hepatocytes.

Fig 5 here

Fig.5. The integrated charges versus cell densities. The charges were integrated current responses from 5s to 45 s.

3.3 Comparison of electrochemical and MTT-based detection of LPS hepatocytotoxicity

To confirm the feasibility of the electrochemical detection method developed in our study, the cytotoxic effect of LPS on rat primary hepatocytes was evaluated using the chronoamperometric method and an MTT assay. Fig.6 shows the viability of hepatocytes in the presence of 0 to 8 μg mL^{-1} of LPS. The results of the chronoamperometric and MTT tests were compared using a paired *t* test. Although the average cell viabilities determined using the MTT assay were lower than those determined using chronoamperometry, no significant difference was observed between the results of the 2 methods.

Fig 6 here

Fig 6 The viability effect of LPS on rat primary liver cells. LPS (10μL) was added to each well to keep the final concerntration in the range of 0~8 μg mL^{-1} for incubation of 12 h.

4. Conclusions

Our results showed that chronoamperometry using ferricyanide and succinate mediators is an efficient, alternative method for rapidly assessing the viability of primary liver cells. The results of the chronoamperometry method are comparable to those of the MTT viability assay. The chronoamperometric viability assessment can be completed in 20 min, and the use of a disposable SPE minimizes protein adsorption and cross contamination. The chronoamperometric method may be useful for distinguishing hepatocarcinoma cells from healthy hepatocytes.

Acknowledgments

This study was supported by the Taiwan Grains and Feeds Development Foundation and the National Science Council, Taiwan (NSC 100-2113-M-040-002). We would like to thank two anonymous reviewers and the editor for their comments.

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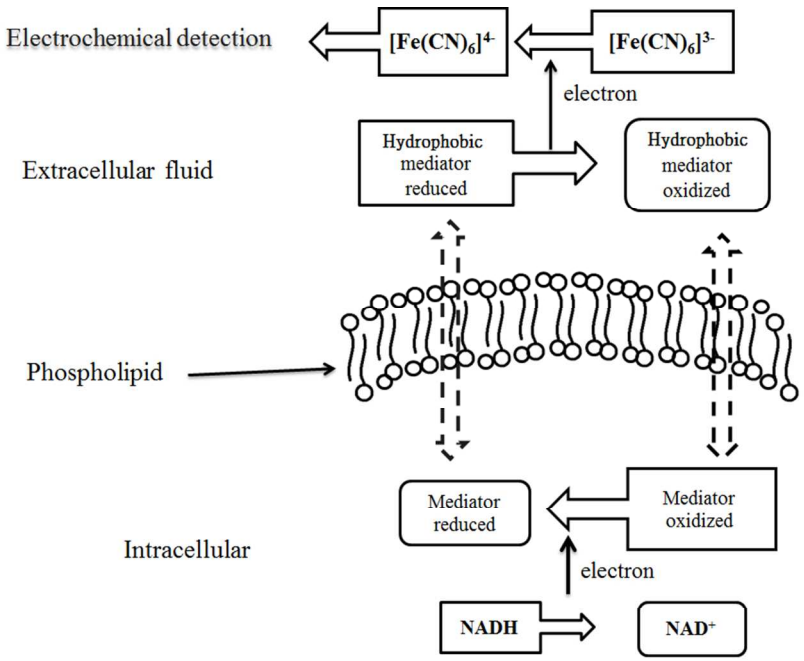

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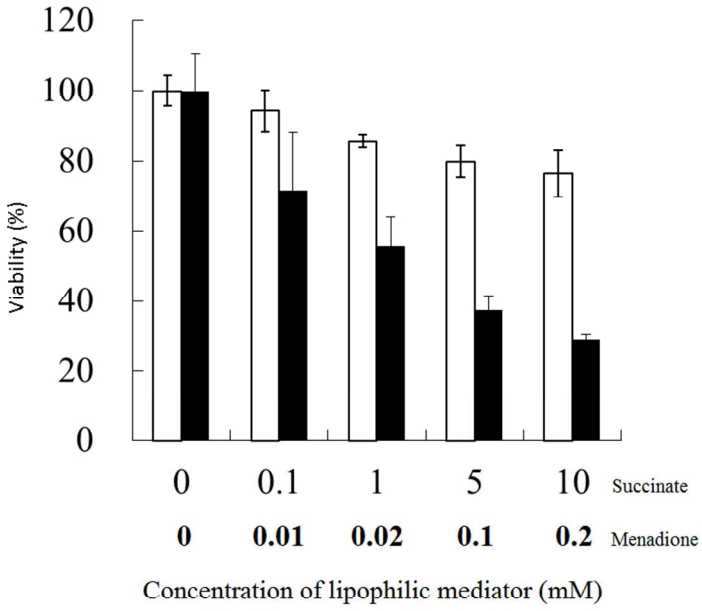

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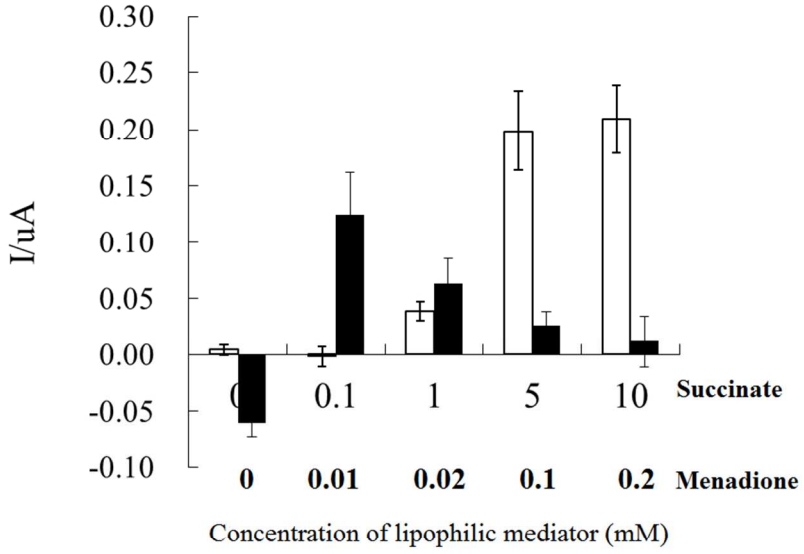

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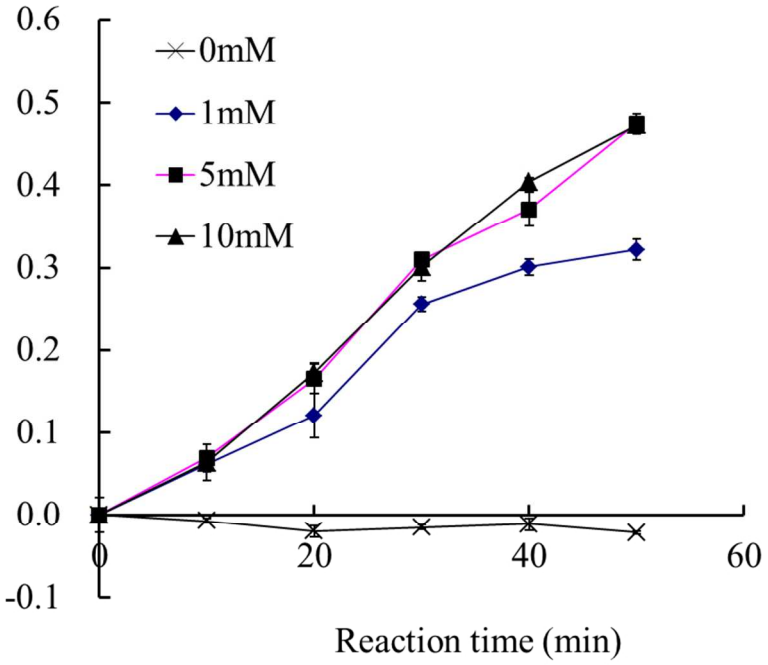

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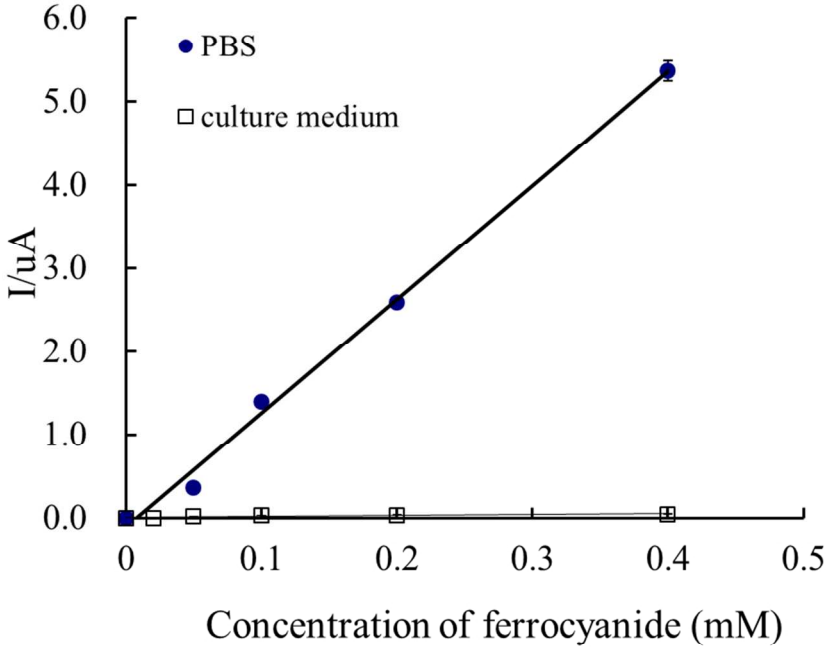

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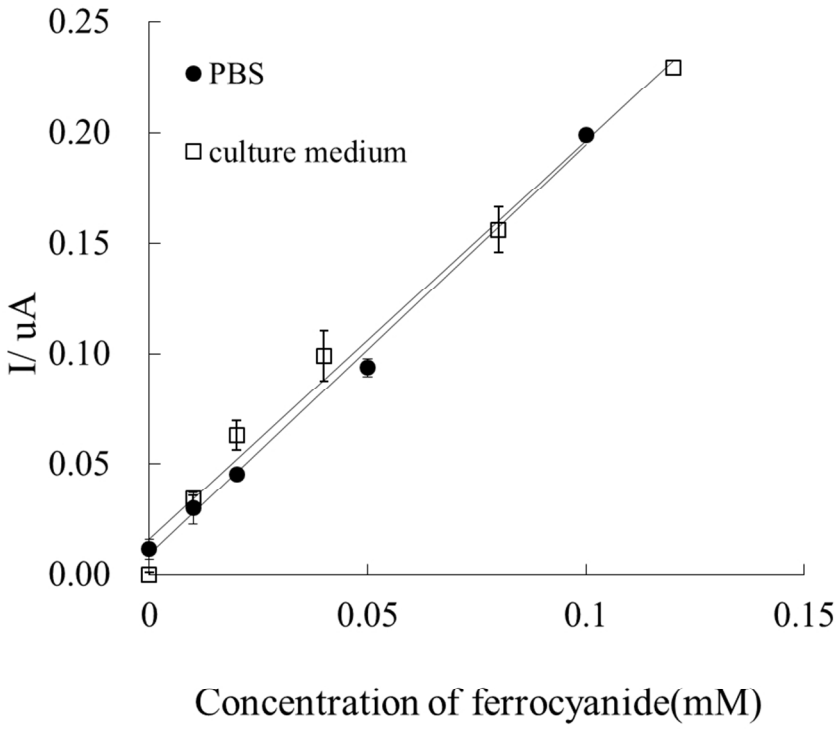

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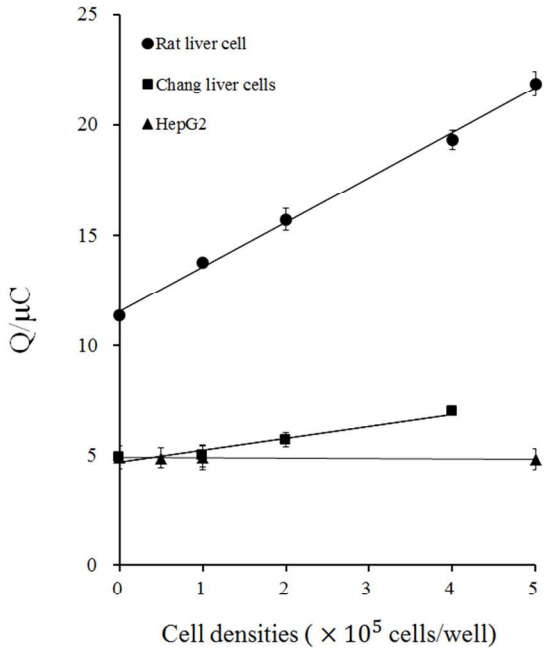

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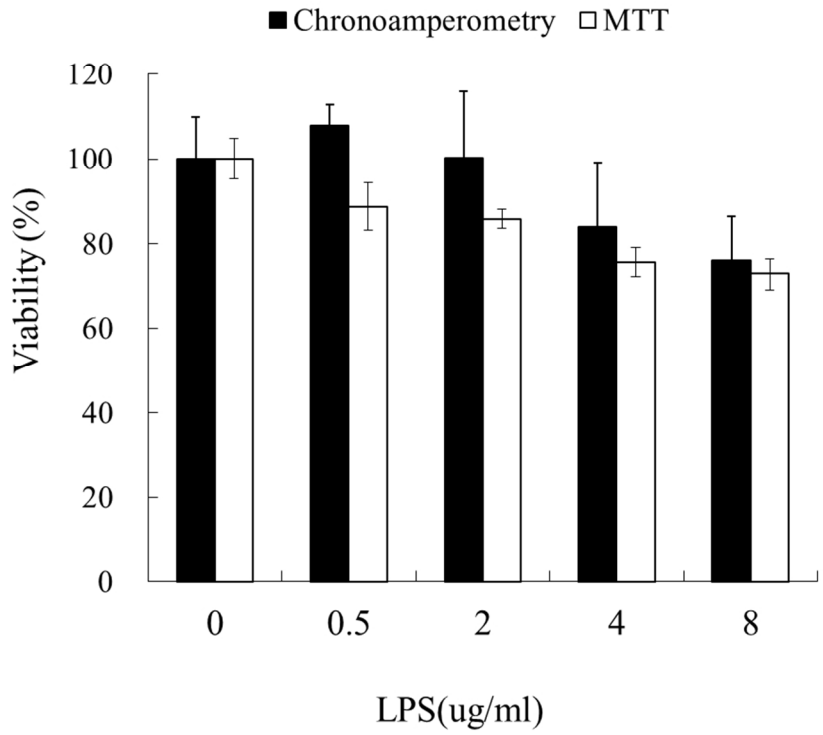

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Chronoamperometry of ferricyanide and succinate mediators is an efficient analytical tool for assessing the viability of primary hepatocytes.