An *in vitro* model for assessment of excitotoxicity induced by glutamate and neuroprotection using digitized fluorescent imaging of mitochondrial potentials

Received: 9/12/2010 Accepted: 28/5/2011

Mohammed Sherwan Muhyaddin*

Abstract

Background and objectives: The established methods to demonstrate the neuroprotective efficacy of drugs still use *in vivo* animal tests. The objective of the present study, therefore, was to develop *in vitro* screening systems based on digitized fluorescent imaging of individual neurons from neuronal cell cultures as a practical model for assessment of neuroprotectors during excitotoxicity .

Methods: Cortical cells from embryonic mice were cultivated on glass coverslips Cells were loaded with the fluorescence dye rhodamine-123. The coverslips were mounted in a temperature controlled flow-through chamber. The superfusion chamber was mounted on an inverted microscope. The emission light after excitation of the dye was measured with an intensified CCD camera, accordingly the changes in the mitochondrial membrane potential were monitored.

Results: Neurotoxic concentrations of glutamic acid induced a rapid and irreversible damage to mitochondrial membrane potential. The non-competitive NMDA-receptor antagonist MK-801 (dizocilpine) preserved neuronal viability. The loss of rhodamine-123 fluorescence highly correlated with the ongoing neuronal cell death and was shown to be a suitable parameter to determine the neuroprotective action of pharmaceutical compounds.

Conclusion: Loss of the mitochondrial membrane potential can be used as dynamic markers of cellular injury in vitro. Presumably, the in vivo animal experiments are required and cannot be avoided completely. However, the described approach can at least enlighten the preliminary neuroprotective effect of drugs or their critical concentrations directly on the neuronal level and by that way avoid a large number of animal tests.

Key wards: neuronal culture, digitized florescent imaging, glutamic acid, neurotoxicity**.**

Introduction

The term excitotoxicity corresponds to damage of cells induced by overactivation of receptors for glutamate, the major excitatory neurotransmitter in the central nervous system. The sustained activation of NMDA (N-methyl-D-aspartate) receptor, a subclass of the ionotropic glutamate receptor family, accounts for the majority of glutamate excitotoxicity.[1] The overactivation is a result of excessive release of glutamate from synaptic terminals and/or glial cells as observed in ischaemic neuronal injury in stroke and seizure induced neuronal injury, neurodegeneration in Alzheimer disease and Parkinson's disease. [2-4] Because neither stroke nor other severe neurodegenerative pathological conditions can presently be treated effectively [5], substantial efforts are being made by pharmaceutical industries to develop drugs which will protect the brain from neurodegeneration that follows a variety of diseases. These efforts now concentrate on the development of drugs which are neuroprotective that is a drug which can be given to minimize the neuronal damage and the consequences

***College of Pharmacy, Hawler Medical University, Erbil, Iraq**

of this damage. Experiments in this field of pharmacology are usually dependent on the development of various animal tests that mimic the neuropathological consequences of excitotoxicity. For example the occlusion of an intracranial artery *in vivo*, is widely used to produce focal brain ischemia in animals, mainly rodents. [6] Making the *in vivo* assay is labor-intensive and expensive. However, although one of the major advances of stroke research has been through the development of reproducible techniques for the induction of focal or global ischemia in animals, the fact remains that there is a substantial number of variants of those *in vivo* bioassays. [7] Thus, the development of experimental strategies that improves the specificity of the assay and saves animal is a challenge. A probable solution to these problems can be an *in vitro* neuronal cell culture system, which allows the monitoring of pharmacological effects in an animal-saving and organ-specific manner. [8] Another advantage of the *in vitro* test is the ability to control and to determine the experimental parameters in a wider range than in the whole animal. Furthermore, and on this basis our understanding of the pathocellular mechanisms of neuronal cell death will be improved the indication of this *in vitro* tests potential may be justified in an early state of drug development. The major objective of the present study, therefore, was to develop *in vitro* assays using digitized fluorescent imaging in which neurons can be studied with respect to changes in the mitochondrial membrane potential which is regarded as predictive markers of neurotoxicity. [9]

Methods

This work has been performed in the laboratories of the Department of Molecular Biology and Cell Culture Technology of the University of Mannheim for Applied Sciences. The methods applied in the work are all based on the standard methods of the department.

Neuronal cell culture

Cortical cell cultures were prepared from embryonic (E15) NMRI mices. Female mices were killed by cervical dislocation and the embryos were placed in PBS (phosphate buffered saline) without calcium and magnesium. Cortical hemispheres were dissected away from the rest of the brain, the meninges were removed and the resulting tissue placed in PBS at room temperature. Cortices were dissociated by incubation in 0.05 % Trypsin in calcium and magnesium free PBS supplemented with 0.02 % EDTA (Ethylenediaminetetraacetic acid) for 15 min at 37 ^{o}C. Trypsin was inactivated by adding serum containing maintenance media consisting of Eagle´s minimal essential media supplemented with 10 % heat inactivated horse serum 2 mM glutamine, 25 mM glucose and 26 mM $NaHCO_{3.}$ The tissue was then triturated 10 times using fire polished pasteur pipettes in maintenance medium followed by an additional trituration with a fire polished pipette with reduced tip diameter for 3 times. After centrifugation (10 min, 1000 x g) cells were resuspended in maintenance medium and viability determined by phase contrast microscopy or by staining a small aliquot with fluorescein diacetate, a vital dye, to adjust viable cell density by counting stained cells under fluorescence optics using a hemocytometer.

Plating on glass coverslips

The cell suspension was diluted to 860,000 cells/ml and 5 ml of the resulting cell suspension was plated onto poly-L-ornithine precoated (15 µg/ml, dissolved in 10 mM sodium tetraborate solution, adjusted to pH 8.4 with HCL, 6-12 h) rectangular 40 x 24 mm or 37,5 x 22 mm glass-coverslips which were housed in Petri dishes (60 mm diameter). Prior to plating, the coated glass cover slips were washed three times with sterile water and incubated at 37^{o} C in plating media supplemented with 10% foetal bovine serum for 1-2 hours. Normally, the dissection of one pregnant mouse with 13-15 embryos resulted in 20-25 plated

coverslips. Cultures were maintained in a humidified 5 % CO_2, 95 % air atmosphere at 37 ^oC. After 7 days in vitro, non neuronal cell division was inhibited by treating the cultures with10 µM uridine and 10 µM (+)-5 -fluo-2´-deoxyuridine for 24 to 48 hours.. After 14-16 days in culture, cells were used for experiments.

Digital imaging of mitochondrial membrane potential

Changes in the mitochondrial membrane potential were monitored with the fluorescence dye rhodamine-123 (rh-123), which accumulated in mitochondria on the basis of their membrane potential. Cells were loaded with 2 µM rh-123 for 15 min at room temperature, washed twice and used for the experiment. After washing with HBSS (Hepes buffered salt solution) the coverslips were mounted in a temperature controlled flow-through chamber(Warner Inc., Science Products, Hofheim, D). The superfusion chamber was mounted on an inverted Zeiss Axiovert 100 TV microscope. Cells were superfused with prewarmed HBSS using a Warner in line heater at a rate of 2 ml/min for 10 minutes prior to starting the experiments. The temperature in the superfusion chamber was measured with a Pt-100 electrode and was set to 37 ^oC. Digital imaging was performed with the IonVision-system equipped with a 75 W xenon excitation light source fitted to the microscope. The excitation wavelength was selected with an electronic filter wheel and excitation light was reduced by neutral density filters. Emission light was measured at 530 nm with an intensified CCD camera (extended ISIS M, Photonic Science, Norwich, UK). Illumination of the cells was restricted to the time of data acquisition by the use of an electronic shutter. Frames were acquired routinely every 40 seconds and stored on an Apple McIntosh Quadra 800 Computer. Higher acquisition rates (up to 1 picture/2sec) were used at time points of special interest, for example during the application of glutamate. After the experiment, defined neurons were selected using custom build software and analyzed individually. For each experiment a number of 20-40 single neurons were analyzed and the resulting fluorescence changes were averaged. Fluorescence changes were normalized to the initial baseline fluorescence determined within the first 2 minutes of data acquisition or fluorescence ratio values were estimated.

Experimental procedures: Digital imaging

Baseline fluorescence in standard HBSS was measured for 10 min and than the solution was switched to an appropriate glutamate containing solution for 5 min. Afterwards the neuronal cell culture was superfused with HBSS for an additional 60 min. Cultures were superfused with dizocilpine containing medium from the beginning of the glutamate treatment throughout the experiment. The superfusion rate of 2 ml/ min was maintained by a roler tube pump. The fluid level within the chamber was adjusted by a variable aspirator device which allowed a higher suction rate than the superfusion rate. Standard fluid content was 250 µl, resulting in a eightfold solution exchange per min.

Determination of cell viability: At the end of each experiment, cells were superfused with HBSS containing 50 µg/ml PI (propidium iodide) for a minimum of 10 min. Fluorescence images were collected after washout of the PI at excitation wavelengths 340 and 490 nm and emission at 530 nm. Afterwards, PI stained cells were related to the individual changes of their fluorescence signals during the experiment to determine specific differences between surviving and dead neurons.

Data analysis and statistics

Custom software was used to transform the raw fluorescence data into display ready data and to perform simple statistical analysis on groups of cultures. Results are expressed as mean ±SD. Statistical differences between experimental data within the same experimental group were calculated by a paired Student's T-test. Values were considered significantly different when $P < 0.05$.

Results

Rhodamine-stained mitochondria of selected cortical cells were located in neurites and somata but concentrated within the annular cytoplasm around the nucleus (figure 1). In this work, only those mitochondria in the perinuclear ring were selected and analyzed. rh-123 is well retained by mitochondria for several hours. Under control conditions there was only a loss of about 5 % of the initial fluorescence after a period of 2 hours. However, that decrease was not statistically significant different when compared to the starting value. Treatment of cortical cell cultures with 300 µM glutamate in the presence of Mg^{2+} (1.3 mM) for 5 minutes resulted in an early rapid increase of the rh-123 fluorescence indicating a depolarization of the mitochondrial membrane potential. Furthermore, glutamate induced mitochondrial depolarization is a unique feature of the selected cortical neurons. In all of the experiments performed all cortical neurons (up to 1000 analyzed neurons) first responded with an increase in the rh-123 fluorescence after a glutamate challenge which was then followed by a long-lasting decrease of the rh-123 fluorescence. After 2 hours, only 14 % of the initial fluorescence value remained which was statistical significant different (t-test, $P < 0.001$) from the fluorescence level at the beginning. It is known, that mitochondria which loose their ability to maintain a normal membrane potential are not able to accumulate rh-123. To elicit if the observed loss of rh-123 fluorescence after the glutamate challenge depends on the disruption of mitochondria, and also is accompanied with an ongoing cell death, we used propidium iodate (PI) at the end of the experiments to visualize dead cells. The single cell analysis revealed two different response pattern of the glutamate induced changes rh-123 fluorescence which was dependent on the later fate of the neurons. As demonstrated in fig.3 all cells positive stained for PI at the end of the experiments showed an early and complete loss of rhodamine 123 fluorescence. In contrast, neurons which excluded PI 2 h after the glutamate challenge maintained 90 % of their initial fluorescence intensity through the duration of the experiment. Therefore a rapid loss of the rh-123 fluorescence correlates highly with an early ongoing excitotoxic neuronal cell death. Glutamate may be neurotoxic by activating multiple receptor subtypes e.g. NMDA, non-NMDA or metabotropic receptors. To determine the contribution of the specific glutamate receptor sub-types to the observed changes in the mitochondrial membrane potential, we performed experiments using subtype specific glutamate receptor antagonist MK-801 which is well known for its selectivity on the NMDA receptor subtype. This is to test the hypothesis that the activation of the NMDA receptor channel is responsible for the observed depolarisation. Co-application of 1 µM MK-801 with 300 µM glutamate completely prevents mitochondrial depolarization and all neurons analyzed survived. When applied together with glutamate.

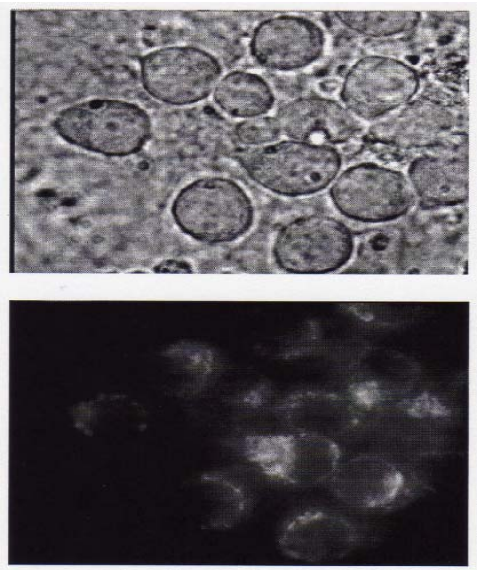

Figure 1: Appearance of cortical neurons using transmitted light and fluorescence microscopy. Cells were loaded with the selective mitochondrial stain rhodamine-123. Magnification was 640x.

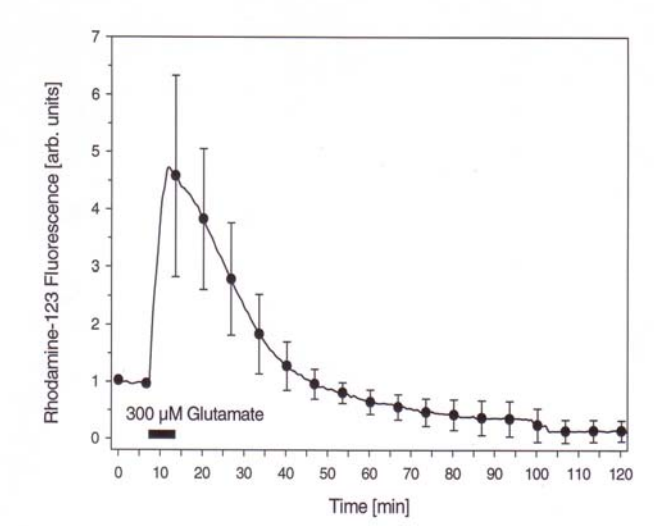

Figure 2: Changes of the mitochondrial membrane potential in cortical neurons induced by glutamate. Means ±SD are from six independent experiments. Rh-123 fluorescence intensity changes are expressed as arbitrary units normalized to the initial value.

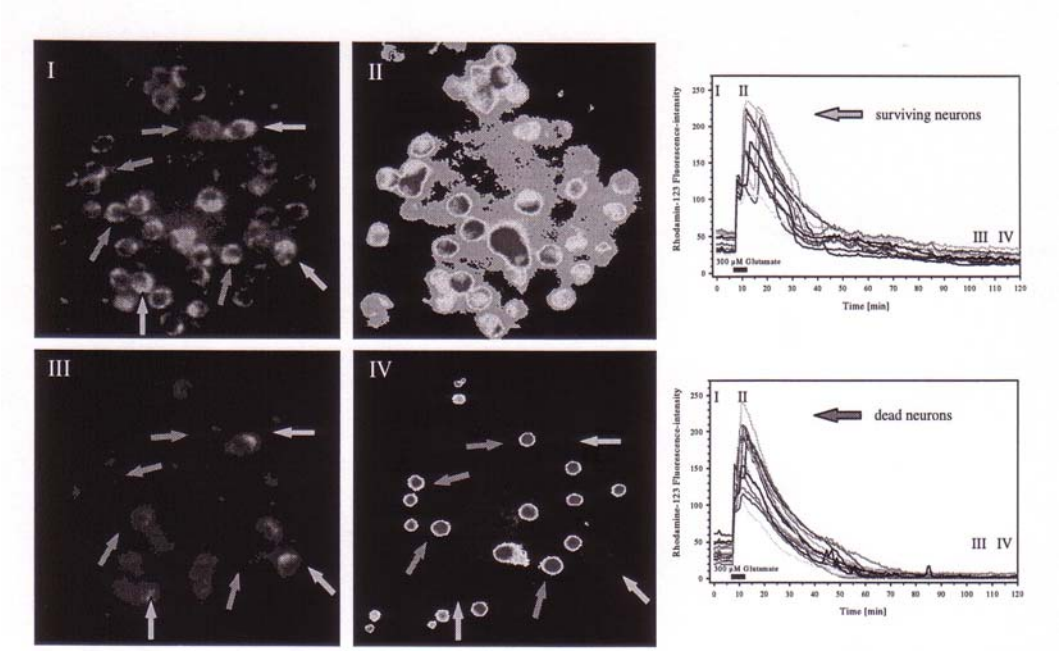

Figure 3: Different patterns of rh-123 fluorescence intensity changes resulting from a 300 µM glutamate challenge. Neurons which stained positive by the vital dye propidium iodate at the end of the experiment have completely lost their rh-123 fluorescence during the experiment. (I) rh-123 loaded cells at t=0 (II) increased rh-123 fluorescence as a response of the glutamate challenge (III) rh-123 fluorescence intensity at the end of the experiment. The fluorescence of some neurons completely disappeared (dark arrows) other maintained rh-123 fluorescence (light arrows) (IV) propidium iodate staining of dead neurons at the end of the experiment.

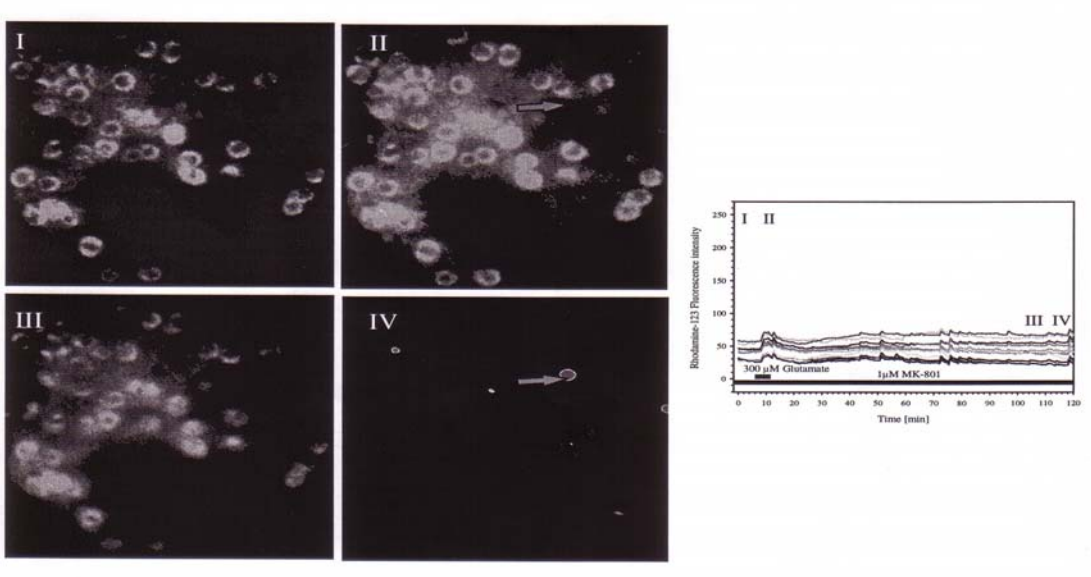

Figure 4: Protective action of MK-801(1 µM) on rh-123 fluorescence intensity changes induced by a 300 µM glutamate challenge. (I) rh-123 loaded cells at t=0, (II) unaffected rh-123 fluorescence as a response of the glutamate challenge, (III) rh-123 fluorescence intensity at the end of the experiment. Note, that no neurons completely disappeared, (IV) PI staining of dead neurons at the end of the experiment. Note, that the stained nucleus (arrow) showed no corresponding rh-123 fluorescence at the beginning of the experiment.

Discussion

The present study provides a mean to perform cytotoxicity experiments at a precisely controlled temperature, taken into account the temperature sensitivity of excitotoxic phenomena. It also serves the ability to study with precision the time course of cell death, which may vary with different insults. The results obtained correlate highly with manual techniques (cell counting) and with other recognized methods to measuring cytotoxicity for example the lactate dehydrogenase efflux method.[10] As shown in our glutamate experiments, this method are applicable in determine the time profile of ongoing neuronal cell death as well as the neuroprotective action of a glutamate receptor channel antagonist in our *in vitro* model which supports *in vivo* findings of delayed neuroprotective actions of NMDA-receptor antagonists. [6] Taken together, in the described model the glutamate induced neuronal cell death is mainly due to the activation of NMDA receptor linked calcium ion channels, this may reflect the early ongoing neuronal cell death and the protection which was found with the selective blocker of the ion channel linked to this receptor. The demonstration of the fact that a short exposure of murine cortical neurons to 300 µM glutamate resulted in a rapid increase of the mitochondrial potential and later it was toxic to neurons. In agreement with the literature our results show that in cultured cortical neurons the loss of mitochondrial membrane integrity and the subsequent loss of the oxidative phosphorylation system may be the reason of the observed inability to maintain calcium homeostasis and the eventual intracellular calcium overload and its consequences in the cell death. [11, 12] Up to date there are many compounds showed the neuroprotective activity both *in vitro* and *in vivo models* of neuronal excitotoxicity. However, none of them proved to be therapeutically useful drug in the prevention of

the outcome of stroke [13, 14], due to many complications resulted in the blockade of NMDA receptors in the brain. The results obtained in the MK-801 and other drugs in animal and human studies showed a small therapeutic window for these compounds.

Acknowledgment

I should extend my sincere thanks and gratitude to Professor Dr. Mathias Hafner in the University of Mannheim for Applied Sciences, Germany for allowing access to his excellent research facilities and his valuable guidance, to Dr Detlev Melzian for his collaboration and help through out this research work.

References

1.Sattler R and Tymianski M. Molecular mechanisms of glutamate receptor-mediated excitotoxic neuronal cell death. Mol Neurobiol , 2001, 24: 107-129.

2..Blandini F., Porter R. H., Greenamyre J, T, Glutamate and Parkinson's disease. Molecular Neurobiology 1996, 12, 73-94

3.Hynd, M. R., Scott, H. L., and Dodd, P. R. Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease. Neurochem. Int. 2004, 45, 583–595.

4.Lynch, D. R. and Guttmann, R, P. Excitotoxicity: Perspectives Based on *N*-Methyl-D-Aspartate Receptor Subtypes, J. Pharmacol. Exp. Ther**.,** 2002; 300: 717

5.Chavez J. C., Hurko O, Barone F C, and Feuerstein G Z, Pharmacologic interventions for stroke: looking beyond the thrombolysis time window into the penumbra with biomarkers, not a stopwatch. Stroke; 2009, 40: 558 - 563.

6.McAuley M.A., Rodent models of focal ischemia, Cerebrovascular and Brain Metabolism Reviews, 7, 152-180, 1995

7.Fox G., Gallacher D., Shevde S., Loftus J.and Swayne G., Anatomic variation of the middle cerebral artery in the Sprague-Dawley rat, Stroke, 1993, 24, 2087-2093

8.Oberpichler-Schwenk H. and Krieglstein J., Primary cultures of neurons for testing neuroprotective drug effects, Journal of Neural Transmission, [Suppl] , 1994, 44, 1-20.

9.,R.J. and Reynolds, I.J., Mitochondrial depolarization in glutamate stimulated neurons: an early signal specific to excitotoxin exposure, Journal of Neuroscience, 1996, 16, 5688-5697.

10.Koh J.Y. and Choi D.W Quantitative determination of glutamate mediated cortical neuronal injury in cell culture by lactate dehydrogenase efflux assay, Journal of Neuroscience Methods,1987 20, 83 -90.

11.Brookes P. S., Yoon Y., Robotham J. L., Anders M. W., and Sheu S., Calcium, ATP, and ROS: a mitochondrial love-hate triangle Am J Physiol Cell Physiol 2004, 287, C817–C833

12.Duchen M. R., Mitochondria and calcium: from cell signalling to cell death J. Physiol*.* 2000, 529, 57-68

13.Rother J., Neuroprotection does not work! Stroke, 2008, 39, 523–524.

14.Faden A. I. and Stoica B., Neuroprotection: Challenges and Opportunities, Arch Neurol., 2007, 64, 794-800