ELSEVIER

Brain Research Protocols 7 (2001) 193–202

BRAIN RESEARCH INTERACTIVE

www.bres-interactive.com

Interactive protocol

Multiple-label immunocytochemistry for the evaluation of nature of cell death in experimental models of neurodegeneration☆

Emil Adamec[a,b,*], Fusheng Yang[c,d], Greg M. Cole[c,d], Ralph A. Nixon[e,f]

[a]*Mailman Research Center, McLean Hospital, 115 Mill Street, Belmont, MA 02478, USA*
[b]*Department of Psychiatry, Harvard Medical School, Boston, MA 02115, USA*
[c]*Sepulveda Veteran's Administration Medical Center, Sepulveda, CA 91343, USA*
[d]*Department of Medicine and Neurology, UCLA, Los Angeles, CA 90095, USA*
[e]*Nathan Kline Institute for Psychiatric Research, Orangeburg, NY 10962, USA*
[f]*Department of Psychiatry and Cell Biology, New York University School of Medicine, New York, NY 10016, USA*

Accepted 20 April 2001

Abstract

A prominent feature of neurodegenerative diseases is a loss of specific neuronal populations. The pathophysiological mechanisms responsible are, however, poorly understood. Primary cultures of rodent embryonic neurons represent a useful experimental system for investigation of molecular pathways of neurodegeneration and mechanisms of cell death. Here, we report a technique utilizing triple-label immunocytochemistry with confocal immunofluorescence detection designed to simultaneously assess multiple parameters of cell injury in individual hippocampal neurons in primary culture. This method combines detection of DNA damage (TUNEL or Klenow assay) with double-label immunocytochemistry for the activated form of caspase-3 or, alternatively, caspase-cleaved actin (fractin), and microtubule-associated protein-2 (MAP-2) or β-tubulin. The combined evaluation of the form of nuclear damage (karyorrhexis, pyknosis), the presence or absence of activated caspase-3, and the extent of the damage to cell cytoskeleton, allows for precise assessment of the extent of injury and the mode of cell death (apoptosis, oncosis) for individual neurons.

Theme: Cellular and molecular biology

Topic: Staining, tracing, and imaging techniques

Keywords: Apoptosis; Oncosis; Necrosis; Caspase-3; Actin; Confocal microscopy

1. Introduction

Common neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, frontotemporal dementias, and others, have as one of the prominent findings a profound loss of neurons. This slowly progressive loss of specific neuronal populations might actually represent the anatomical and functional substrate of many clinical symptoms associated with these diseases. The pathophysiological mechanisms and the molecular pathways involved are, however, poorly understood. In general, the molecular pathway of cell death characterized by somatic swelling and karyolysis or pyknosis has been in the past called necrosis, while the pathway resulting in cell shrinkage and karyorrhexis is termed apoptosis. Recently, the Society of Toxicologic Pathologists recommended use of the term necrosis to describe the finding of dead cells in histological sections, regardless of the pathway involved, and the term oncosis to name the pathway characterized by cell swelling [15]. Based on this recommendation, this report, therefore, uses the terms apoptotic necrosis and oncotic necrosis, respectively.

An essential component of experimental studies targeted towards the understanding of basic molecular mechanisms of neurodegeneration is the availability of an experimental system in which the relevant conditions can be tested and reliably evaluated. Primary cultures of rodent embryonic hippocampal neurons represent a useful in vitro system for this type of studies [1–4]. The goal of this study was to

☆Published on the World Wide Web on 31 May 2001.

*Corresponding author: Tel.: +1-617-855-3804; fax: +1-617-855-3479.

E-mail address: eadamec@mercury.mclean.org (E. Adamec).

1385-299X/01/$ – see front matter
PII: S1385-299X(01)00072-1

develop paradigms of experimental neuronal injury in vitro that model the processes involved in neurodegeneration in vivo, and to establish methods for the characterization of molecular pathways involved in cell death. Here, we report a multiple-label protocol combining the use of DNA damage assays together with double-label immunocytochemistry that allows the mode of cell death to be established unequivocally in individual neurons.

2. Type of research

- Primary neuronal cultures.
- Molecular models of neurodegeneration.
- Immunocytochemistry.
- Confocal microscopy.

3. Time required

Half-day for cell culture preparation (autoclaving, coating of coverslips). Half-day for the culture itself. The length of maintaining the cultures in vitro depends on the design of the actual studies, here 13–21 days. The length of the exposure of neurons to drugs will again vary, here 4 h to 3 days. Two days for immunocytochemical labeling. Approximately 2–3 days for confocal microscopy, depending on the number of conditions employed in a study. On average, one experiment employing multiple drugs, evaluated with both phase-contrast, brightfield, and confocal microscopy will take about 4 weeks to complete.

4. Materials

4.1. Animals

CD1 time-pregnant rats were maintained and used in this study in accordance with the guidelines described in the *Guide for the Care and Use of Laboratory Animals* from the US Department of Health and Human Services (National Institute of Health, Bethesda, MD). All measures were made to minimize pain and discomfort. The protocol for preparation of primary cultures was approved by the Institutional Animal Care and Use Committee (IACUC) at McLean Hospital (Belmont, MA).

4.2. Special equipment

- Autoclave for sterilization of dissection equipment and glass coverslips.
- Dissection microscope and dissection hood for the preparation of primary cultures.
- Tissue culture incubator and tissue culture hood.
- Upright Leica DM LS microscope (Leica Microsystems, Deerfield, IL).
- Inverted Leica DM IL microscope with phase-contrast objectives.
- True-color Spot digital camera (Diagnostic Instruments, Sterling Heights, MI).
- TCS NT confocal microscope with Ar, Kr, and He–Ne lasers (Leica).
- Macintosh G3 computer with Photoshop software (Adobe Systems, San Jose, CA).

4.3. Cell culture reagents

- Neurobasal culture medium, B27 supplement, glutamine, penicillin, streptomycin (Life Technologies, Gaithersburg, MD).
- Fetal calf serum and horse serum (HyClone Laboratories, Logan, UT).
- Poly-D-lysine (MW 30 000–70 000) from Sigma (St. Louis, MO).
- Cytosine β-D-arabinofuranoside (ara-c) from Sigma.
- Tissue culture multi-well plates (Corning Costar, Cambridge, MA).
- Glass coverslips D type (Bellco, Vineland, NJ).

4.4. Drugs

- Staurosporine, camptothecin, menadione, glutamate, glycine (Sigma).
- Dizocilpine maleate (MK-801), 6,7-dinitroquinoxaline-2,3-dione (DNQX) from Research Biochemicals International (Natick, MA).
- 3-Morpholinosydnonimide (SIN-1) from Molecular Probes (Eugene, OR).

4.5. Supplies for immunocytochemistry

- Paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA).
- Goat serum (Jackson ImmunoResearch, West Grove, PA).
- Saponin, Fast Red (Sigma).
- Tris-buffered saline 20× stock (Amresco, Solon, OH).
- DNA dye Sytox (Molecular Probes).
- Ribonuclease (RNase) from Boehringer Mannheim (Indianapolis, IN).
- Triton X-100 10% (Calbiochem, La Jolla, CA).
- Hybridization trays (Sigma).
- Hybridization coverslips (Molecular Probes).
- Gill's hematoxylin #3 (Fisher, Pittsburgh, PA).
- GelMount, CrystalMount (Biomeda, Foster City, CA).

4.6. Primary antibodies

- Mouse monoclonal antibody to microtubule-associated protein 2 (MAP-2) from Boehringer-Mannheim (Indianapolis, IN).

- Mouse monoclonal antibody to β-tubulin isotype III (Sigma).
- Rabbit polyclonal antibody CM1 to activated caspase-3 (IDUN Pharmaceuticals, La Jolla, CA). This antibody recognizes the p18 subunit of cleaved caspase-3, but not the zymogen [23].
- Rabbit polyclonal antibody to caspase-cleaved actin (fractin) from G. Cole, Sepulveda VA Medical Center (Sepulveda, CA). This antibody was prepared by immunizing rabbits with a synthetic peptide K-YELPD representing the last five amino acids of the C-terminus of the 32-kDa actin fragment produced during apoptosis [28].

4.7. Secondary antibodies

- Goat anti-mouse IgG and goat anti-rabbit IgG secondary antibodies, and streptavidin, both tagged with either Alexa Fluor 488 or 568 (Molecular Probes).
- Goat biotinylated anti-rabbit IgG, goat anti-mouse IgG tagged with Cy5, streptavidin-Cy3, goat anti-mouse FITC (Jackson ImmunoResearch) [Note: In initial experiments, anti-mouse IgG tagged with FITC was used. This secondary antibody was, however, later replaced with secondary antibodies tagged with Alexa Fluor 488 or Cy5 [17]. These fluorophores are generally brighter than FITC and have better resistance to photobleaching during confocal microscopy. Also, the spectral properties of streptavidin-Alexa Fluor 568 make it better suited for multiple labeling than streptavidin-Cy3.]

4.8. DNA damage assays

- TUNEL (Trevigen, Gaithersburg, MD).
- Klenow (Trevigen).

5. Detailed procedures

5.1. Primary culture of rat hippocampal neurons

Establishment of the cultures of rat embryonic (days 18–19) hippocampal neurons was based on the method of Brewer et al. [7], with minor modifications, as previously described [1,2,4]. Dissected hippocampi were triturated with a fire polished Pasteur pipet in phosphate-buffered saline (PBS, 10 mM) and the resulting mostly single-cell suspension was added to the Neurobasal medium supplemented with B27 (50×), glutamine (2 mM), glutamate (25 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), fetal calf serum (1%), horse serum (1%), and plated at the density of approximately 200–300 cells/mm^2 on poly-D-lysine (50 μg/ml) pre-coated glass coverslips (diameter 12 mm) in 24-well plates. The cultures were maintained in a humidified atmosphere of 5% CO_2–95% air at 37°C in a tissue culture incubator. Ara-c (1 μM) was added at 4–6 days after plating to prevent the proliferation of non-neuronal cells. The cells were fed at 7 days in vitro and subsequently once a week by the addition of 25% of the starting culture medium volume with fresh Neurobasal/B27 mixture. The cultures contained approximately 10–30% of glial cells.

5.2. Drug treatments

The experiments were performed on hippocampal neurons 13–21 days in culture. Drugs were added as sterile stock solutions directly into the original culture medium. Staurosporine and camptothecin were prepared as 1000× stock solution in dimethyl sulfoxide (DMSO) and diluted with the Neurobasal medium to 100×. Menadione was prepared as 1000× stock in water. Glutamate and glycine were 100× stock solutions in water. SIN-1 was 100× stock solution in PBS. In order to prevent secondary toxicity caused by glutamate released from dying neurons, the administration of staurosporine, camptothecin, menadione, and SIN-1 was done in the presence of the NMDA receptor antagonist MK-801 (1 μM) and the non-NMDA receptor antagonist DNQX (10 μM). Control cultures received the same antagonists and the corresponding drug vehicle solution. All treatments were performed at 37°C in a tissue culture incubator.

5.3. Immunocytochemistry with confocal fluorescence detection

The primary hippocampal neurons on glass coverslips were fixed in paraformaldehyde (4%) in PBS for 30 min at room temperature, permeabilized by 30-min incubation with Triton X-100 (0.1%) in Tris-buffered saline (TBS, 25 mM), and subsequently blocked for 30 min with TBS supplemented with goat serum (1%) and saponin (0.1%) for 30 min (TBS-saponin). The TBS-saponin buffer was also used for the incubation of all primary and secondary antibodies. Primary antibodies were incubated overnight at 4°C in a hybridization chamber filled with a small amount of water. To reduce the amount of a primary antibody required for each labelling procedure, the following method was used. Into each hybridization chamber, six glass slides were placed and each was covered with a 22×22-mm hybridization coverslip. Approximately 50–75 μl of the solution containing a primary antibody were then pipetted on each of the hybridization coverslips. Finally, glass coverslips containing primary neurons were inverted and gently placed on the drop of the solution. Primary antibodies were used in the following concentrations: MAP-2 1:500, β-tubulin 1:1000, CM1 1:4000, fractin 1:500. The fluorescence-tagged secondary antibodies were incubated for 1 h at room temperature. The secondary antibodies tagged with Alexa Fluor 488, 568, or Cy5 were used at 1:500. To label nuclear DNA, the solution with

secondary antibodies also contained the nucleic acid dye Sytox (100 nM) [16] and RNase (1:100–1:200). In between each step, the coverslips were washed 3×5 min with TBS. Coversliping was done with GelMount.

5.4. Immunocytochemistry for brightfield microscopy

The fixation, permeabilization, blocking, and incubation of primary antibodies was done as described above. Biotinylated anti-rabbit secondary antibody (1:500) was incubated for 1 h at room temperature, followed by streptavidin tagged with alkaline phosphatase (1:500, 1 h), and developed with Fast Red (5 min). The coverslips were lightly (15 s) counterstained with hematoxylin. Coverslipping was done with CrystalMount.

5.5. Combined DNA damage assay and two primary antibodies with detection with confocal microscopy

For the detection of DNA damage combined with immunocytochemistry, the DNA damage assays TUNEL [10] or Klenow [12,26] were performed first, according to the manufacturer's instructions, as described previously [4,5]. For both assays, the cells were first fixed in paraformaldehyde (4% in PBS) for 30 min, followed by permeabilisation with TBS-saponin for 30 min. For the TUNEL assay, the cells were incubated with a reaction mixture containing the enzyme terminal deoxynucleotidyl transferase and biotinylated dNTP nucleotides for 1 h at 37°C. For the Klenow assay, the cells were incubated with a solution containing the Klenow enzyme and biotinylated dCTP. In the second step, the cells were exposed to primary antibodies in TBS-saponin overnight at 4°C. The next day, the incorporation of the biotinylated nucleotides was detected with streptavidin Alexa Fluor 568 (1:500), the primary antibodies were detected with goat secondary antibodies tagged with Alexa Fluor 488 and Cy5 (both 1:500), respectively, in TBS-saponin for 1 h at room temperature. Streptavidin and both secondary antibodies were applied in the same solution. Coverslipping was done with GelMount. The images were acquired with a Leica TCS NT confocal system connected to an inverted microscope. Each individual image was averaged by eight to 16 passes of the scanner. The images in Fig. 2 and Fig. 4F represent projections of individual sequential sections (typically eight sections, 1 μm apart) into a composite image. The images in Fig. 4D,E represent single sections through the middle of the nucleus. Omission of primary antibodies in immunocytochemical staining or replacement of labeling enzymes with distilled water in DNA damage assays reduced the signal intensity to a background level. Also, in experiments involving multiple labeling, control experiments were performed to verify that the secondary antibodies did not recognize each other. When only one primary antibody was applied followed by the detection with a mixture of both anti-mouse and anti-rabbit secondary antibodies, no signal was detected for the secondary antibody lacking the primary.

6. Results

The objective of this study was to establish a reliable method for the assessment of the mode of cell death in individual neurons under conditions of experimental, drug-induced, neurodegeneration. Apoptotic necrosis was induced by the administration of either camptothecin, a DNA topoisomerase I inhibitor and a DNA damaging agent [13,19], or staurosporine, a potent, but nonspecific inhibitor of protein kinases [20]. Oncotic necrosis was modeled by the administration of high doses of either glutamate, a potent agonist at both the NMDA and the non-NMDA glutamate receptors, or SIN-1, a NO donor.

The morphological features of cell injury were initially assessed by phase-contrast microscopy (Fig. 1). Administration of camptothecin or staurosporine led to a gradually developing toxicity with morphological features of cell shrinkage and fragmentation of neuritic processes. On the other hand, glutamate or SIN-1 caused rapidly developing toxicity with massive somatic swelling and pronounced damage to neuritic processes. All neurons with these morphological features were dead, as evidenced by compromised membrane permeability to trypan blue [4].

Camptothecin and staurosporine are well characterized drugs for the induction of apoptotic necrosis [13,19,20,24]. Similarly, glutamate or SIN-1 (in high doses) can be considered model drugs for the induction of oncotic necrosis. When evaluating the mode of toxicity for a less well characterized compound, it is preferable to be able to assess multiple parameters of injury for each individual cell. Multiple-label confocal microscopy is well suited for that purpose. An example of such an approach is shown in Fig. 2. To assess karyorrhexis or pyknosis, DNA was labeled with an intercalating dye Sytox. An antibody to either the activated form of caspase-3 (CM1) or, alternatively, an antibody to caspase-cleaved actin (fractin) was used to provide evidence of caspase-3 activation. Damage to dendrites or to both dendrites and axons was visualized by immunocytochemistry for MAP-2 or β-tubulin, respectively. Alternatively, double-stranded DNA breaks were labeled with the TUNEL assay or single-stranded breaks with the Klenow assay. Examples of the apoptotic pathway of neuronal death in Fig. 2A show perfect association of karyorrhexis with evidence for caspase-3 activation. Due to the high sensitivity of MAP-2 to proteolytic degradation, no MAP-2 immunoreactivity can be detected in neurons with nuclear segmentation. Neurons that underwent oncotic necrosis (Fig. 2B) had pyknotic nuclei, positive for either the TUNEL or the Klenow DNA damage assays. These neurons were also invariably negative for activated caspase-3 [4] or fractin (Fig. 3).

Even though the multiple-labeling approach described

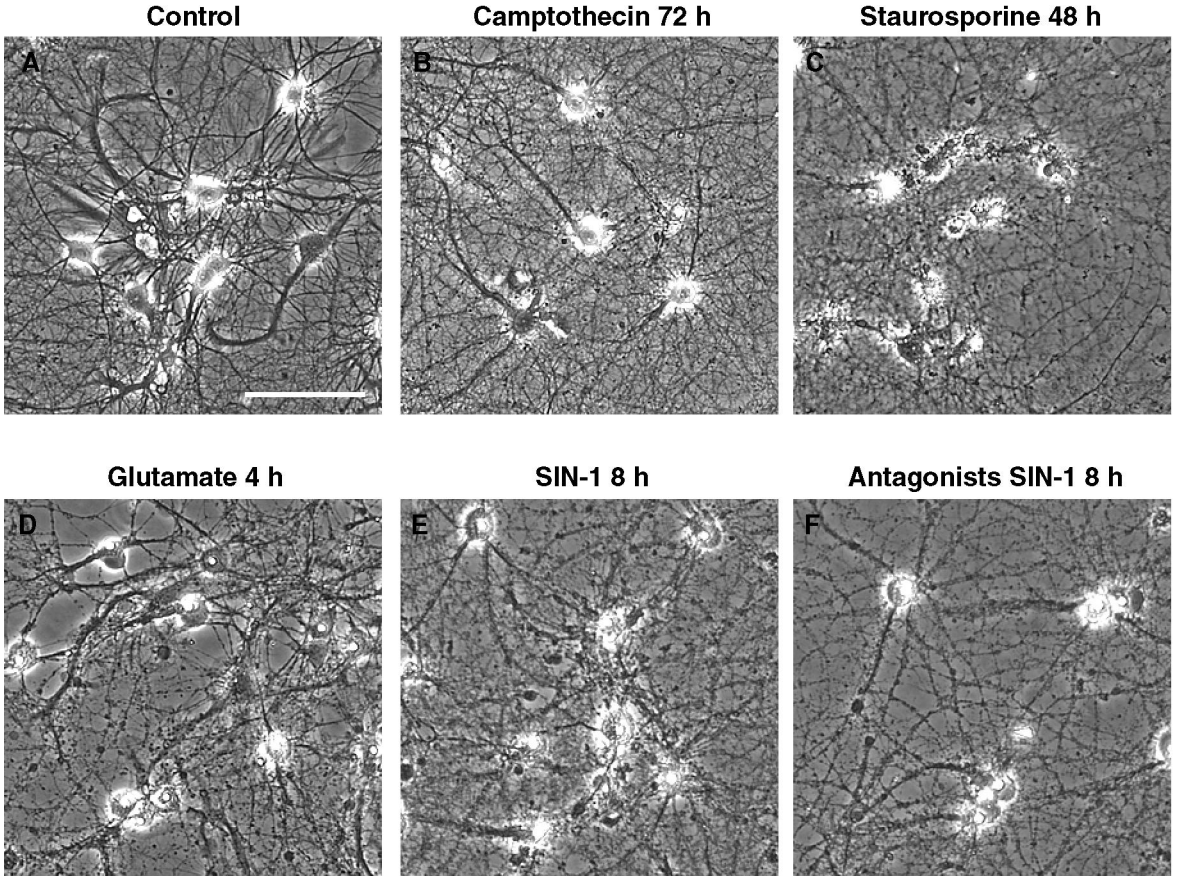

Fig. 1. Evaluation of drug-induced toxicity with phase-contrast microscopy. Rat hippocampal neurons 13–21 days in vitro were exposed to (A) vehicle alone (DMSO, 0.1%) in the presence MK-801 (1 μM) and DNQX (10 μM), (B) camptothecin (1 μg/ml) in the presence MK-801 and DNQX, (C) staurosporine (1 μM) in the presence MK-801 and DNQX, (D) glutamate (100 μM, supplemented with 10 μM glycine), (E) SIN-1 (1 mM), and (F) pre-incubated with MK-801 and DNQX for 1 h followed by SIN-1 (1 mM). The images were acquired with a Spot digital camera connected to an inverted microscope. Scale bar=100 μm for all.

above allows precise assessment of the mode of cell death in individual neurons, in certain circumstances, there might be a need to evaluate a large number of experimental conditions faster than what is possible with confocal microscopy. For this purpose, we have establish a protocol which uses nuclear labeling with hematoxylin combined with immunocytochemistry for the activated form of caspase-3 or fractin (Fig. 3). Nuclear fragmentation was again associated with the presence of activated caspase-3 in the models of apoptotic necrosis (Fig. 3A–C), while pyknotic nuclei in the models of oncosis were negative for activated caspase-3 (Fig. 3D–I). The detection with alkaline phosphatase is very sensitive and the Fast Red substrate provides excellent contrast with hematoxylin counterstain facilitating rapid cell counting.

The usefulness of the multi-parametric labeling in evaluation of the mode of neuronal death can be demonstrated by an example shown in Fig. 4. In this experiment, hippocampal cultures were subjected to oxidative stress [22] by treatment with menadione, a drug which undergoes redox cycling and depletes cellular antioxidants [14,21,25]. High doses of menadione caused rapidly developing toxicity with signs of somatic swelling, representing the oncotic pathway of necrosis (Fig. 4B). Treatment with low doses of menadione represented, however, an example of a more complex situation. Some neurons died by the apoptotic pathway (Fig. 4 D), some by the oncotic pathway (Fig. 4 E). In some instances, it was possible to encounter both situations in the same visual field (Fig. 4F). The TUNEL assay labeled both apoptotic and oncotic neurons. Fractin labeling specifically identified neurons that underwent the apoptotic pathway of cell death. MAP-2 immunocytochemistry identified the remaining surviving neurons (both TUNEL and fractin negative). In a large number of sections, it was, therefore, possible to determine both the overall toxicity associated with this drug treatment and the percentage of neurons undergoing apoptosis versus oncosis.

7. Discussion

There are practical issues involved in obtaining satisfactory results with a multi-parametric assessment of the

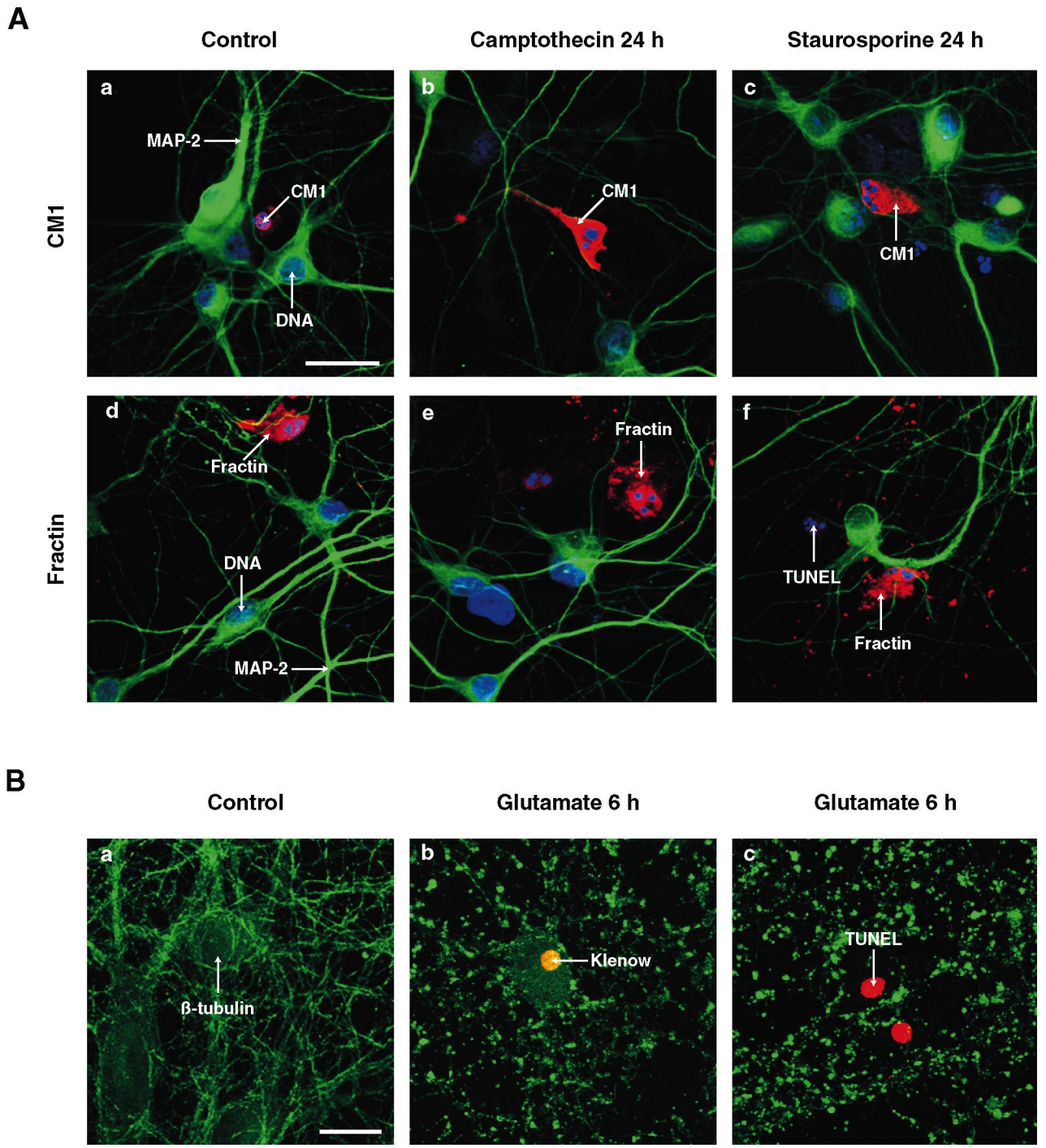

Fig. 2. Evaluation of drug-induced toxicity with multiple label confocal immunofluorescence. (A) Apoptotic pathway of neuronal death. Rat hippocampal neurons were exposed to camptothecin or staurosporine, both in the presence of MK-801 and DNQX. Triple label confocal immunofluorescence was done as described in Section 5. CM1 or fractin were labeled with Alexa 568, MAP-2 with Cy5. In (a–e), DNA was labeled with Sytox. In (f), the DNA damage assay TUNEL was performed first (detected with streptavidin-Alexa 568), followed by double labeling with MAP-2 (Cy5) and fractin (Alexa 488). Scale bar=50 μm in all. (B) Oncotic pathway of neuronal death. Hippocampal neurons were exposed to glutamate. The DNA damage assays Klenow (a,b) or TUNEL (c) were performed first (streptavidin-Cy3), followed by β-tubulin immunocytochemistry (FITC). Scale bar=10 μm.

mode of cell death, especially when evaluating compounds of unknown mechanism and mode of action. Two initial optimization steps are especially important.

The first step requires the establishment and optimization of a reliable experimental system, which, in this case, involves primary cultures of rat hippocampal neurons. We have slightly modified the culture protocol of Brewer [6], originally developed for serum-free conditions,

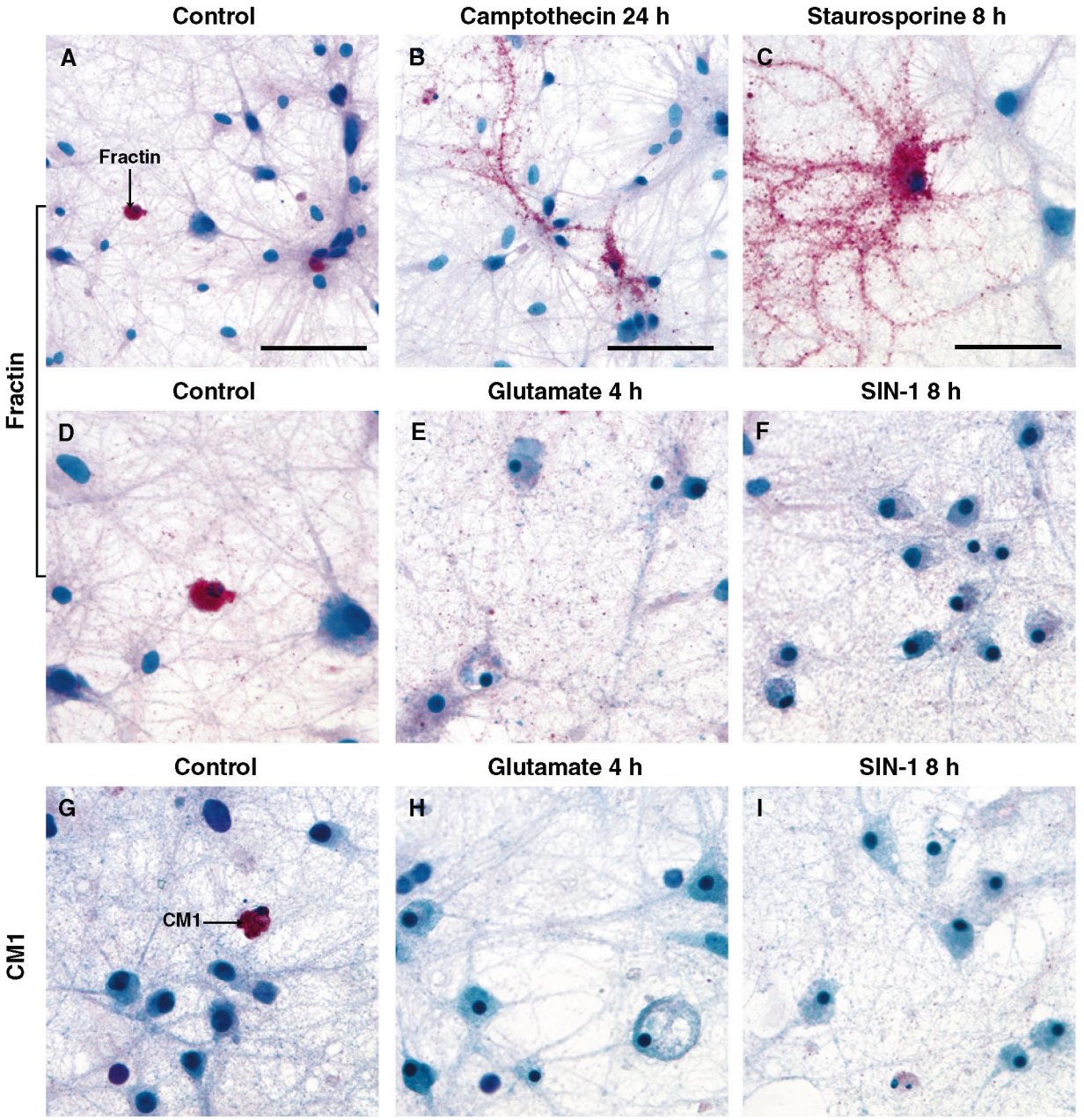

Fig. 3. Evaluation of drug-induced toxicity with brightfield microscopy. The drug doses were as indicated in the legend for Fig. 1. Fractin (A–F) and CM1 (G–I) were detected with Fast Red. Light hematoxylin counterstain. The images were acquired with a Spot digital camera connected to an upright microscope. Scale bar: (A,B)=100 μm; (C–I)=50 μm.

to include a low concentration of serum (1%) in the culture medium. This procedure generally improves cell viability and responsiveness to drug effects, even though it occurs at the expense of having a higher percentage of astrocytes present. Astrocytes, however, can be readily distinguished by morphological features or by GFAP immunocytochemistry.

The second concern involves the drug treatments themselves. Nearly pure cultures of hippocampal neurons are very sensitive to the actions of glutamate. Since glutamate might be released by exocytosis during drug-induced depolarization of glutamatergic neurons or by membrane leakage from dying neurons, it might cause secondary toxicity (usually oncosis) superimposed on the effect of the drug of interest [18]. Routinely, therefore, we initially test any drug effect both alone and in the presence of glutamate receptor antagonists.

The morphological features of drug-mediated toxicity should be initially evaluated with phase-contrast microscopy. This allows rapid and inexpensive assessment of the drug effect on the appearance of cell soma (shrinkage versus swelling) and neuronal processes (fragmentation).

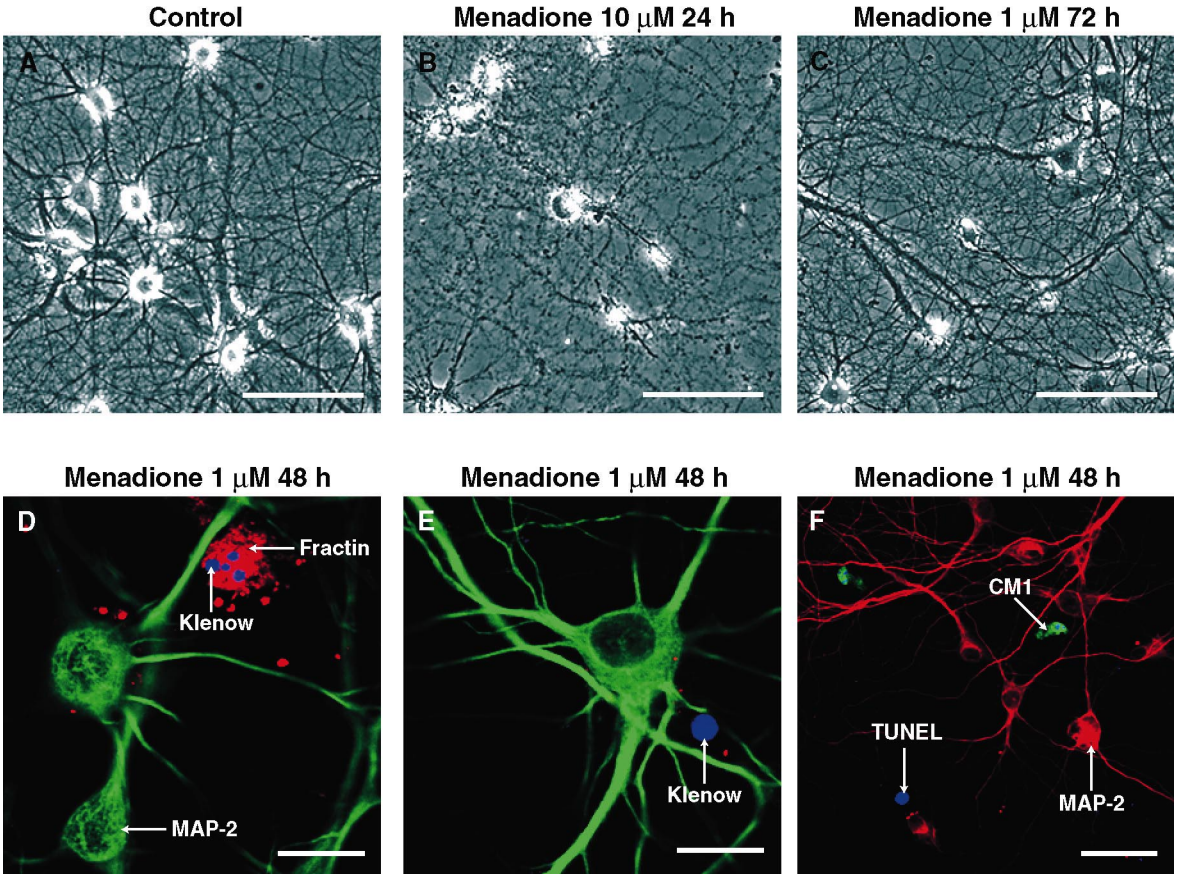

Fig. 4. Oxidative stress-induced toxicity in primary hippocampal neurons. Menadione was administered in the presence of MK-801 and DNQX. Control cultures received vehicle solution in the presence of MK-801 and DNQX. (A–C) Phase contrast images. (D–F) Confocal immunofluorescence. In (D) and (E), the DNA damage assay Klenow (streptavidin-Alexa 568) was performed first, followed by immunocytochemistry for fractin (Alexa 488) and MAP-2 (Cy5). In (F), the DNA damage assay TUNEL (streptavidin-Alexa 568) was performed first, followed by immunocytochemistry for CM1 (Alexa 488) and MAP-2 (Cy5). Scale bar: (A–C)=100 μm; (D, E)=25 μm; (F)=50 μm.

Even though preliminary, initial assignment of the mode of cell death (apoptosis versus oncosis) can be made. Quantification of the extent of cell death can be done by combining this approach with trypan blue staining [1,4].

The best method for evaluating the mode of cell death for each individual neuron is multi-parametric immunocytochemistry, especially when combined with confocal microscopy. The number of parameters that can be evaluated simultaneously depends on how many fluorophores can be simultaneously imaged, which is determined by the configuration of the confocal system itself. DNA labeling with an intercalating dye, such as Sytox in this study, is used to visualize nuclear damage, such as segmentation or pyknosis. Since RNA would also be labeled, RNase digestion is recommended. This approach is quite inexpensive, but since all DNA will be labeled, dead neurons will not immediately stand out in a visual field. This can be achieved by labeling DNA strand breaks with either the TUNEL or the Klenow assay. Even though the positivity of labeling with either of these two assays does not, by itself, allow one to distinguish the apoptotic and the oncotic pathways of cell death [8,11], the assays are nevertheless valuable in visualizing all dead neurons. In some paradigms of cell injury, the Klenow assay becomes positive earlier than the TUNEL assay [9], however, for labeling of end-stage neurons, both assays are equivalent. Also, since both assays incorporate biotinylated nucleotides that are detected with streptavidin-Alexa Fluor 568 (or comparable red fluorophore), this leaves the widely spectrally separated green (488 nm) and far-red (633 nm) channels for the detection of antibodies. The wide separation is especially important if issues of colocalization are being addressed [27]. Since the DNA damage assays detect all dead neurons, the subset which died by apoptosis is distinguished by labeling with an antibody to activated caspase-3 or fractin. MAP-2 immunocytochemistry will label surviving neurons.

Finally, based on the objectives of a particular study, this protocol can be modified to directly address further specific needs. Frequently, it will be desirable to interface an additional antibody of interest with a DNA damage assay. We would recommend detecting the antibody of interest with Alexa Fluor 488 or Cy2, both of which are very bright and resistant to photobleaching. The TUNEL

(or Klenow) assay is detected with streptavidin-Alexa Fluor 568. Since there is quite good correlation between karyorrhexis and apoptosis, and pyknosis and oncosis (established previously), the nuclear morphology itself will be indicative of the pathway of cell death. The surviving neurons can detected by either MAP-2, tubulin, or tau protein immunocytochemistry and Cy5.

8. Quick procedures

8.1. Primary hippocampal culture

- Dissect hippocampi from 18–19-day-old rat embryos.
- Triturate 10–15× in PBS (2 ml) with a fire-polished Pasteur pipet.
- Adjust the volume with PBS so that the final plating density equals 200–300 cells/mm^2.
- Add into Neurobasal medium supplemented with B27 (50×), glutamine (2 mM), glutamate (25 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), fetal calf serum (1%), and horse serum (1%).
- Plate on pre-coated glass coverslips, 0.5 ml medium with cells/1 well of a 24-well plate.
- Add ara-c (1 μM) at days 4–6.
- Feed with 25% of the starting culture medium volume at day 7 and subsequently once a week.

8.2. Immunocytochemistry with confocal fluorescence detection

- Fix primary neurons on glass coverslips with paraformaldehyde (4%) in PBS for 30 min.
- Permeabilize with Triton X-100 (0.1%) in TBS for 30 min.
- Block with goat serum (1%) in TBS supplemented with saponin (0.1%) for 30 min. Use this solution also for the incubation of both primary and secondary antibodies.
- Incubate primary antibodies at 4°C overnight.
- Incubate with a mixture of secondary antibodies (typically, one will be tagged with Alexa Fluor 568, the other with Cy5, 1:500), Sytox (100 nM), and RNase (1:100) for 1 h.
- Coverslip with GelMount.
- In between each step, wash 3×5 min with TBS. All incubations are at room temperature unless otherwise stated.

8.3. Immunocytochemistry for brightfield microscopy

- Fix, permeabilize, block, and incubate primary antibody as described above.
- Incubate with biotinylated secondary antibody (1:500) for 1 h.
- Incubate with streptavidin tagged with alkaline phosphatase (1:500) for 1 h.
- Develop with Fast Red for 5 min.
- Counterstain with hematoxylin for 15 s.
- Coverslip with CrystalMount.
- In between each step, wash 3×5 min with TBS.

8.4. Combined DNA damage assays and two primary antibodies with detection with confocal microscopy

- Fix and permeabilize as described above.
- Incubate with either the TUNEL mixture or the Klenow mixture for 1 h at 37°C.
- Incubate with a mixture of primary antibodies (different species) overnight at 4°C.
- Incubate with a mixture of streptavidin-Alexa Fluor 568 (1:500) and two different species secondary antibodies, one tagged with Alexa Fluor 488 (1:500), the other with Cy5 (1:500), for 1 h.
- Coverslip with GelMount.
- In between each step, wash 3×5 min with TBS.

9. Essential literature references

[23,28]

Acknowledgements

This work was supported by grants from the National Institute on Aging, AG00764 and AG05134.

References

[1] E. Adamec, M.L. Beermann, P.A. Nixon, Calpain I activation in rat hippocampal neurons in culture is NMDA receptor selective and not essential for excitotoxic cell death, Brain Res. Mol. Brain Res. 54 (1998) 35–48.
[2] E. Adamec, M. Didier, R.A. Nixon, Developmental regulation of the recovery process following glutamate-induced calcium rise in rodent primary neuronal cultures, Brain Res Dev Brain Res 108 (1998) 101–110.
[3] E. Adamec, M. Mercken, M.L. Beermann, M. Didier, R.A. Nixon, Acute rise in the concentration of free cytoplasmic calcium leads to dephosphorylation of the microtubule-associated protein tau, Brain Res. 757 (1997) 93–101.
[4] E. Adamec, P.S. Mohan, A.M. Cataldo, J.P. Vonsattel, R.A. Nixon, Up-regulation of the lysosomal system in experimental models of neuronal injury: implications for Alzheimer's disease, Neuroscience 100 (2000) 663–675.
[5] E. Adamec, J.P. Vonsattel, R.A. Nixon, DNA strand breaks in Alzheimer's disease, Brain Res. 849 (1999) 67–77.
[6] G.J. Brewer, Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus, J. Neurosci. Res. 42 (1995) 674–683.
[7] G.J. Brewer, J.R. Torricelli, E.K. Evege, P.J. Price, Optimized

survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination, J. Neurosci. Res. 35 (1993) 567–576.
[8] C. Charriaut-Marlangue, Y. Ben-Ari, A cautionary note on the use of the TUNEL stain to determine apoptosis, Neuroreport 7 (1995) 61–64.
[9] M. Didier, S. Bursztain, E. Adamec, L. Passani, R.A. Nixon, J.T. Coyle, J.Y. Wei, S.A. Berman, DNA strand breaks induced by sustained glutamate excitotoxicity in primary neuronal cultures, J. Neurosci. 16 (1996) 2238–2250.
[10] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell Biol. 119 (1992) 493–501.
[11] B. Grasl-Kraupp, B. Ruttkay-Nedecky, H. Koudelka, K. Bukowska, W. Bursch, R. Schulte-Hermann, In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note, Hepatology 21 (1995) 1465–1468.
[12] S. Iseki, DNA strand breaks in rat tissues as detected by in situ nick translation, Exp. Cell Res. 167 (1986) 311–326.
[13] S.H. Kaufmann, Cell death induced by topoisomerase-targeted drugs: more questions than answers, Biochim. Biophys. Acta 1400 (1998) 195–211.
[14] L. Ko, T. Odawara, S.H. Yen, Menadione-induced tau dephosphorylation in cultured human neuroblastoma cells, Brain Res. 760 (1997) 118–128.
[15] S. Levin, T.J. Bucci, S.M. Cohen, A.S. Fix, J.F. Hardisty, E.K. LeGrand, R.R. Maronpot, B.F. Trump, The nomenclature of cell death: recommendations of an ad hoc Committee of the Society of Toxicologic Pathologists, Toxicol. Pathol. 27 (1999) 484–490.
[16] T. Matsuzaki, T. Suzuki, K. Fujikura, K. Takata, Nuclear staining for laser confocal microscopy, Acta Histochem. Cytochem. 30 (1997) 309–314.
[17] N. Panchuk-Voloshina, R.P. Haugland, J. Bishop-Stewart, M.K. Bhalgat, P.J. Millard, F. Mao, W.Y. Leung, Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates, J. Histochem. Cytochem. 47 (1999) 1179–1188.
[18] Z. Pang, J.W. Geddes, Mechanisms of cell death induced by the mitochondrial toxin 3-nitropropionic acid: acute excitotoxic necrosis and delayed apoptosis, J. Neurosci. 17 (1997) 3064–3073.
[19] Y. Pommier, P. Pourquier, Y. Fan, D. Strumberg, Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme, Biochim. Biophys. Acta 1400 (1998) 83–105.
[20] U.T. Ruegg, G.M. Burgess, Staurosporine, K-252 and UCN-01: potent but nonspecific inhibitors of protein kinases, Trends Pharmacol. Sci. 10 (1989) 218–220.
[21] N. Sata, H. Klonowski-Stumpe, B. Han, D. Haussinger, C. Niederau, Menadione induces both necrosis and apoptosis in rat pancreatic acinar AR4-2 J cells, Free Radic. Biol. Med. 23 (1997) 844–850.
[22] N.A. Simonian, J.T. Coyle, Oxidative stress in neurodegenerative diseases, Annu. Rev. Pharmacol. Toxicol. 36 (1996) 83–106.
[23] A. Srinivasan, K.A. Roth, R.O. Sayers, K.S. Shindler, A.M. Wong, L.C. Fritz, K.J. Tomaselli, In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system, Cell Death Differ. 5 (1998) 1004–1016.
[24] L. Stefanis, D.S. Park, W.J. Friedman, L.A. Greene, Caspase-dependent and -independent death of camptothecin-treated embryonic cortical neurons, J. Neurosci. 19 (1999) 6235–6247.
[25] H. Thor, M.T. Smith, P. Hartzell, G. Bellomo, S.A. Jewell, S. Orrenius, The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells, J. Biol. Chem. 257 (1982) 12419–12425.
[26] J.H. Wijsman, R.R. Jonker, R. Keijzer, C.J. van de Velde, C.J. Cornelisse, J.H. van Dierendonck, A new method to detect apoptosis in paraffin sections: in situ end-labeling of fragmented DNA, J. Histochem. Cytochem. 41 (1993) 7–12.
[27] F.G. Wouterlood, J.C. Van Denderen, N. Blijleven, J. Van Minnen, W. Hartig, Two-laser dual-immunofluorescence confocal laser scanning microscopy using Cy2- and Cy5-conjugated secondary antibodies: unequivocal detection of co-localization of neuronal markers, Brain Res. Brain Res. Protocol 2 (1998) 149–159.
[28] F. Yang, X. Sun, W. Beech, B. Teter, S. Wu, J. Sigel, H.V. Vinters, S.A. Frautschy, G.M. Cole, Antibody to caspase-cleaved actin detects apoptosis in differentiated neuroblastoma and plaque-associated neurons and microglia in Alzheimer's disease, Am. J. Pathol. 152 (1998) 379–389.