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APOPTOSIS IN CULTURED SPINAL CORD SLICES OF NEONATAL MOUSE*

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Abstract – Organotypic spinal cord slices from neonatal mammals could be a powerful model for evaluation of cell survival but also cell death mechanisms. The aim of this study was to establish an in vitro model for investigating cell survival and mechanism involved in cell death in neonatal spinal cord slices. The spinal cord was sliced and incubated into culture medium. The MTT assay was carried out to assess the viability of the slices and fluorescent staining was used to study morphological features of apoptosis, where as nucleosomal DNA fragmentation was detected using agarose gel electrophoresis. The results of the present study demonstrated that the slices could be maintained in culture up to 14 days. Both neurons and glial cells died by apoptosis and application of a general caspase inhibitor neither affected slice survival nor nucleosomal DNA fragmentation after 24 h in culture. In addition, the inhibitor failed to block apoptosis in neurons and glial cells in the cultured slices. Our results suggest that in the cultured slices, apoptosis is the main reason for neuron and glial cell death, which occurs by a caspase-independent mechanism.

Keywords – Apoptosis, MTT assay, neonatal mouse, spinal cord slices

1. INTRODUCTION

Organotypic cultures of spinal cord slices from mammalian fetal could be a powerful tool for studies of motor neuron regeneration. These slices have been shown to survive for an extended period of time in culture. Recent studies have shown that both neuronal survival and axonal outgrowth in culture are age dependent. Mouveroux et. al [1], for instance, reported robust neurites outgrowth in cultured slices of lumber spinal cord from one-day-old rats, while no axonal outgrowth occurred from p7 slices. In accordance with this, adult spinal cord slices are notably difficult to maintain in culture and rapidly deteriorate in vitro [1]. However, models in which neonatal and adult spinal cord are used would be very useful for studies of spinal cord injury, evaluation of cell survival, and neuronal degeneration. We have recently developed a model for adult spinal cord slices to investigate mechanisms by which motor neurons die in culture [2]. However, such model has not been developed for neonatal spinal cord slices. The aim of this study is to establish such model for evaluating cell survival and mechanisms involved in cell death.

Cell death in traumatic injuries due to both spinal cord compression and contusion is associated with apoptosis in neurons and glial cells [3]. Apoptosis might be responsible for cell death during spinal cord injury in vitro [4]. The exposure of cultured spinal cord slices to glutamate and nitric oxide also cause apoptosis in neuronal degeneration [5]. Thus, in most cases where the spinal cord slice is subjected to an insult, apoptosis seems to be a dominating mechanism by which cells die. We have also showed apoptosis in motor neurons of the adult spinal cord at the early time point of slice culture [6]. To our knowledge, however, no evidence has been found to show cell death in neonatal spinal cord slices unless the slices are

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exposed to an insult.

Apoptosis is distinguished from other forms of cell death by both morphological and biochemical characteristics [7]. It is believed that apoptosis is mediated by the activation of caspases (Ca^{2+}-independent proteases), which in these cases, the caspase inhibitors suppress apoptosis [8]. However, it has been proven that the inhibition of these proteases does not affect apoptosis in all cases. Thus, this evidence suggests caspase-independent mechanisms for execution of apoptosis [9].

This study was organized to establish a model to investigate the survival of cultured spinal cord slices from neonatal mice and attempts to determine the mechanism causing cell death in such culture.

2. MATERIALS AND METHODS

a) Animal model and preparation of organotypic spinal cord slices

Animals were housed at a 12 h light/dark cycle with water and food *ad libitum*. Organotypic slice cultures were prepared from 7-day-old NMRI mice. The mouse pups were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (60 mg/kg) and subsequently killed by heart puncture. The spinal cord was removed by dissection and placed in ice cold phosphate-buffered saline (PBS, pH 7.4). The thoracic region of the spinal cord [2] was cut transversally into 500 µm thick slices using a McIlwain tissue chopper (Stoelting, USA). The slices were placed in four-well sterile, plastic culture dishes. Each well contained 450 µl medium composed of a mixture of 50% minimum essential medium, 25% Hanks balanced salt solution, 25% horse serum, 25 mM HEPES and penicillin-streptomycin (pH 7.3-7.4). The slices were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

b) Assessment of cellular viability

The MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay was used to assess viability, which is the respiratory capacity of the spinal cord slices. A stock solution of MTT (Sigma, USA) was prepared by dissolving 5 mg/ml in PBS. For the MTT assay, 50 µl of the stock solution was added to the culture medium in each well. The plates were then incubated at 37°C for 20 min. The slices were fixed in a solution of 4% paraformaldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. The slices were then transferred to 200 µl dimethylsulfoxide (DMSO, Merck, Germany) to extract and dissolve the purple formazan crystal. Subsequently, the slices were removed from the extraction mixture and the purple color was measured by spectrophotometry at 492 nm. Cell survival was calculated as the ratio between the optical density of the cultured sample and the mean of the optical density of freshly prepared slices (0 h).

c) Fixation and sectioning

Freshly prepared (0 h) and cultured slices were fixed in Stefanini's fixative (2% paraformaldehyde, 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2) for at least 2 h. The fixed slices were washed in PBS (3×5 min) and incubated overnight in 20% sucrose in PBS at 4°C. The slices were then frozen in a Tissue Tek (Miles, USA) embedding medium and cut into 10 µm-thick sections in a cryostat. The sections were collected and mounted on Poly-L-lysine coated glass slides.

d) Assessment of apoptosis

Morphological features of apoptosis were determined by staining with a combination of propidium iodide (PI, Sigma, USA, 10 µg/ml in PBS, 15 min at room temperature) and nuclear stain Hoechst 33342 (Sigma, USA, 10 µg/ml in PBS, 1 min at room temperature). The cryostat sections were washed in PBS (3×5 min), mounted in glycerol/PBS (1:1) and coverslipped. Motor neurons were identified by

morphological criteria (large cell bodies and large nuclei) and position (ventral horn). Glial cells, astrocytes and oligodendrocytes, were identified by immunostaining (see below). Apoptotic cells displayed cell shrinkage, nuclear and chromatin condensation. Digital photographs were taken with an Olympus camera attached to an Olympus fluorescence microscope (Olympus Optical Co Ltd, Japan) using the appropriate excitation and emission filters at 400 × magnification.

Agarose gel electrophoresis was performed to detect nucleosomal DNA fragmentation. To this end, DNA from fresh (0 h) or cultured spinal cord slices were extracted using a genomic DNA purification kit according to the manufacturers protocol (Promega, USA). Equal amounts of the extracts from each sample were loaded on a 2% agarose gel (ICN, USA) containing ethidium bromide (ICN, USA, 0.5 μg/ml). Electrophoresis was run at 60V for 1 h. Ethidium bromide-stained DNA in the gel was photographed under UV light.

e) Immunohistochemistry

To identify two types of glial cells, the cryostat sections were washed in PBS (3×5 min) and incubated with a 1:3000 dilution of rabbit glial fibrillary acidic protein (GFAP) antibody (Dako, Denmark, a marker for astrocytes) or 1:1500 dilution of rabbit myelin basic protein (MBP) antibody (Chemicon, UK, a marker for oligodendrocytes) in a moist chamber at 4°C overnight. After washing in PBS (3×5 min), the sections were exposed to a goat anti rabbit Alexa 488 (Molecular Probes, USA) labeled secondary antibody at room temperature for 1 h. The sections were then washed in PBS (3×5 min), counterstained with Hoechst 33342, mounted in glycerol/PBS solution (1:1) and coverslipped.

f) Drug

N-Benzyloxycarbonyl-Val-Ala-Asp (O-Me) fluoromethyl ketone (Z-VAD.fmk), a general caspase inhibitor, was purchased from Sigma, USA and dissolved in DMSO. The inhibitor was prepared as a stock solution and stored as aliquot at -20°C. The stock solution was directly added to the medium. Controls received a corresponding amount of DMSO.

g) Statistical analysis

Results are expressed as mean±SD. One way analysis of variance (ANOVA) was used to assess the statistical significance of the data. $P<0.05$ was considered significant.

3. RESULTS

a) Cell viability in the cultured spinal cord slices

In preliminary experiments we examined how long the slices from neonatal mice can successfully survive in culture. MTT assay showed that the cultured slices could be maintained in culture for up to 2 weeks (Fig. 1). At 24 h after culturing, 80 % of the cells in the slices remained viable, while the corresponding figure was 40 % on day 14.

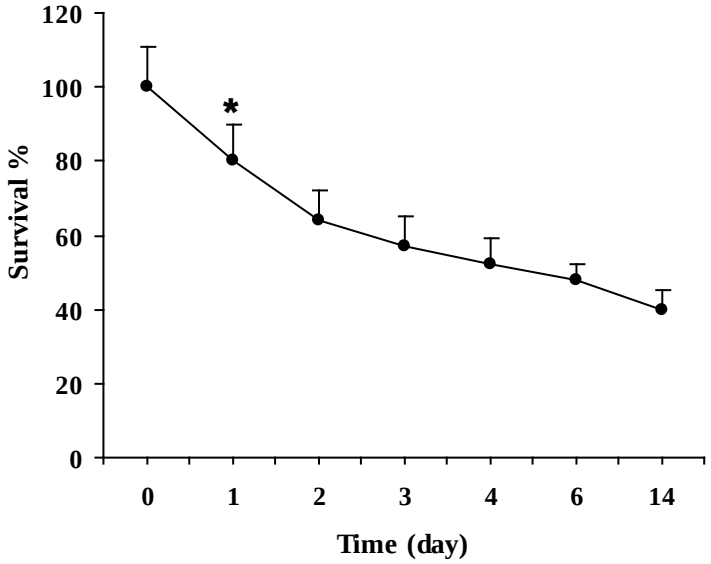

Fig. 1. The time course of slices survival in culture. The MTT assay was used to evaluate the survival of cultured spinal cord slices as a function of time. Mean ±SD, n=8.
* P<0.05 compared with earlier time point (0 h)

b) Neurons and glial cells die by apoptosis

In freshly prepared slices (0 h), both neurons and glial cells displayed large and bright nuclei, the expected distribution of nuclear materials and no apoptotic signs could be observed within the motor neurons (Fig. 2A), interneurons (data not shown), astrocytes (Fig. 2C) and oligodendrocytes (Fig. 2E). After 24 h in culture, the motor neurons (Fig. 2B), astrocytes (Fig. 2D), interneurons (data not shown) and oligodendrocytes (Fig. 2F) showed clear nuclear and chromatin condensation. During the 14 day period both neurons and glial cells also revealed the nuclear apoptotic changes (data not shown).

To further characterize apoptosis, agarose gel electrophoresis was used to detect the appearance of nucleosomal DNA fragmentation in the cultured slices. Extracted DNA from freshly prepared slices (0 h) exhibited only high molecular weight DNA (Fig. 2G, lane 2), whereas DNA from 24 h-cultured slices displayed clear 180 bp fragments of DNA (Fig. 2G, lane3).

c) Apoptosis is induced by caspase-independent mechanism

The findings that cell death occurred through apoptosis prompted us to investigate if caspases participate in the apoptosis observed in this experiment. For this reason, the slices were treated with Z-VAD.fmk (100 µM) for 24 h. The inhibitor did not affect slice survival after 24 h as assessed with MTT (Fig. 3A), nucleosomal DNA fragmentation (Fig. 3B. lane 4) and nuclear and chromatin condensation in the neurons as well as the glial cells (data not shown).

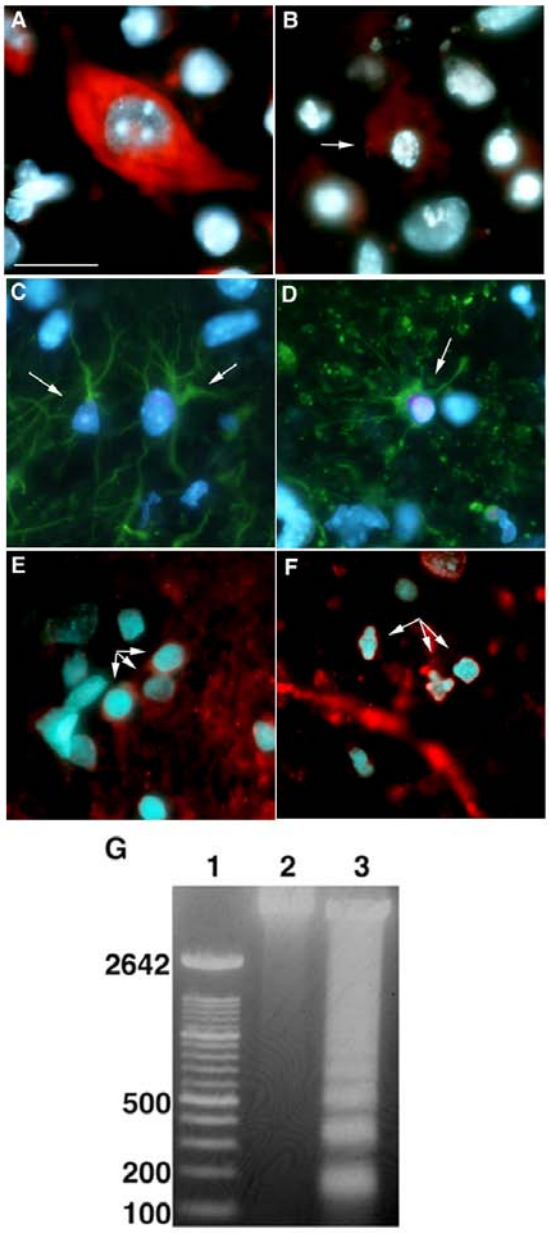

Fig. 2. Morphological features of apoptosis in motor neurons and glial cells. (A and B): Propidium iodide (red) and Hoechst 33342 (blue) staining revealed morphological changes in the nuclei of motor neurons of the cultured slices after 24h. (A) Normal motor neuron. (B) Nuclear and chromatin condensation in motor neuron. (C and D): Astrocytes were stained with glial fibrillary acedic protein (GFAP, green) antibody and counterstained with Hoechst (blue). (C) Normal astrocytes at 0 h. (D) Nuclear and chromatin condensation in astrocytes after 24 h. (E and F): Oligodendrocytes were stained with myelin basic protein (MBP, red) and counterstained with Hoechst (blue). (E) Normal oligodendrocytes at 0 h. (F) Nuclear and chromatin condensation in oligodendrocytes after 24 h. Scale bar 20 µm. (G) Isolated DNA from freshly prepared and cultured slices was analyzed by agarose gel electrophoresis. No nucleosomal DNA fragmentation was noted at time of 0 h (lane 2), whereas nucleosomal DNA fragmentation (DNA laddering) was clear in DNA extracted from slices cultured for 24 h (lane 3). Lane 1: markers expressed in base pairs.

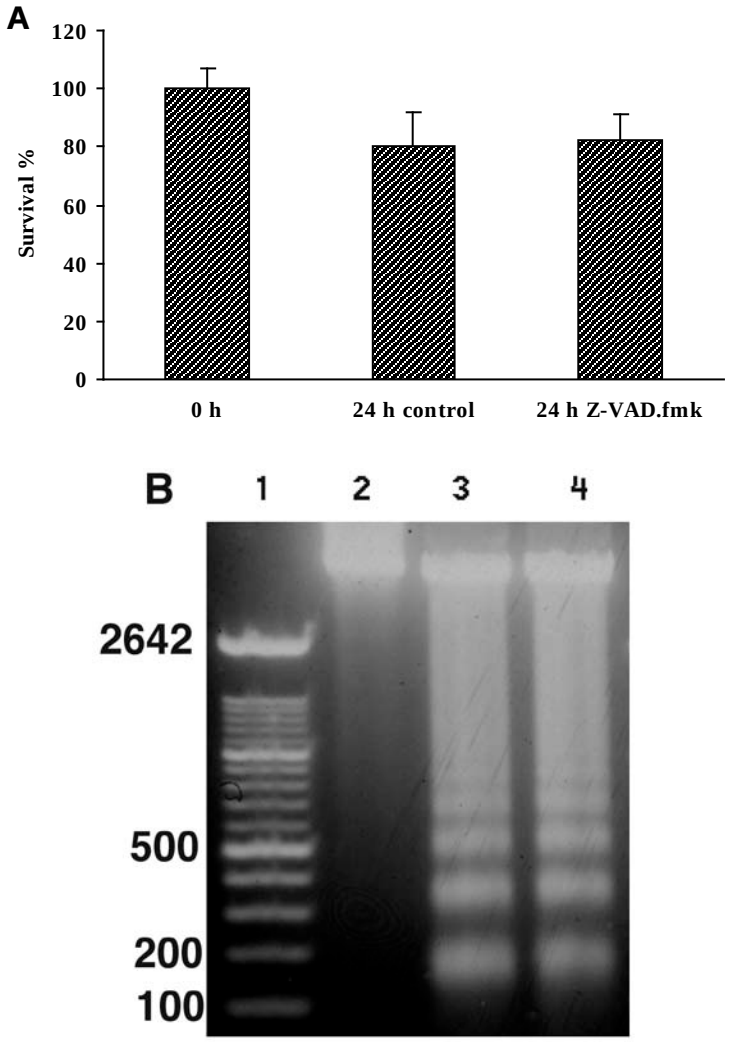

Fig. 3. (A and B): The effect of general caspase inhibitor on cultured slices. The slices were treated with Z-VAD.fmk (100 µM) for 24 h. (A) The MTT assay showed that Z-VAD.fmk was unable to improve slice survival after 24 h. Mean ±SD, n=8. (B) Z-VAD.fmk did not inhibit nucleosomal DNA fragmentation (lane 4) as compared to control (lane 3). Lane 1: Markers expressed in base pairs. Lane 2: 0 h.

4. DISCUSSION

In the present study organotypic spinal cord slices from 7 day old mice were used to evaluate cell survival and mechanism by which cell death occurs in theses slices. We showed that such slices could be maintained in culture up to two weeks. We also found that both neurons and glial cells died by apoptosis. Surprisingly, apoptosis appeared to occur through a caspase-independent mechanism. To the best of our knowledge, this is the first report which shows viability and caspase-independent apoptosis in unprovoked spinal cord slices from P7 mice in culture.

The mitochondrial capacity of the cultured spinal cord slices to reduce MTT as a measure of survival was used. This method, which results in the formation of purple formazan precipitate, is widely used to assess cell survival and proliferation [10, 11]. The method measures mitochondrial integrity, but it should be remembered that MTT reduction can also occur by dehydrogenases outside of the mitochondria [12]. Still, the MTT assay is a convenient method to assess cell viability in brain and spinal cord slices, and it can also be used to localize areas of cell death within the slices [13].

Using nuclear staining by Hoechst 33342, we found that both neurons and glial cells in the cultured slices died by apoptosis. The binding of the fluorescent dye Hoechst 33342 to DNA allows researchers to visualize nuclear and chromatin condensation for qualitative determination of cellular apoptosis [14]. In accordance with this, classical signs of apoptosis including nuclear and chromatin condensation could be observed in the motor neurons, interneurons, astorcytes and oligodendrocytes in the cultured slices. To confirm apoptosis, DNA electrophoresis was used to visualize the occurrence of nucleosomal DNA fragmentation (DNA laddering), a hallmark of apoptosis [15], on agarose gel electrophoresis. The apoptotic nuclear changes are conceivably due to both DNA fragmentation and proteolysis of key nuclear polypeptides [16]. Endogenous endonucleases activities appear to be responsible for producing both large and small DNA fragments. The large fragments are associated with chromatin condensation and form at an early stage of apoptosis of rat thymocyte before nucleosomal DNA fragmentation [17,18]. The nuclear matrix (nuclear skeleton) is composed of several proteins including lamins, NuMA (nuclear mitotic apparatus protein) and poly (ADP-ribose) polymerase (PARP). These proteins can be cleaved during apoptosis [19]. Rapid proteolysis of the nuclear matrix proteins has been suggested as being responsible for chromatin condensation during apoptosis [20], leading to the detachment of the nuclear membrane from the chromatin [16], and the breakdown of nuclear architecture [21]. It is reasonable to assume that the nuclear changes in the cells of the cultured slices are due to the activity of nuclease(s) and protease(s).

Caspases are a family of Ca^{2+}-independent cysteine proteases that have been implicated as key effector molecules in the execution of apoptosis in central nervous system injuries [22]. The application of caspase inhibitors such as Z-VAD.fmk suppresses apoptosis in caspase-dependent models [23], however, there is accumulating evidence indicating the existence of caspase-independent mechanisms [9]. To determine whether the caspase inhibitor could prevent apoptosis in the cultured slices, a broad caspase inhibitor, Z-VAD.fmk was examined. The application of this inhibitor neither affected cell survival nor nucleosomal DNA fragmentation. In addition, the inhibitor failed to prevent nuclear and chromatin condensation in neurons and glial cells. Taken together, these results provide strong evidence that apoptosis in the cultured slices occurs via a caspase-independent mechanism. There are at least two such pathways: Apoptosis inducing factor [24] and endonuclease G [25]. Recently, we have also shown [2,6] that calpains (Ca^{2+}-dependent proteases) play an important role in caspase-independent apoptosis of motor neurons in adult spinal cord slices and that Z-VAD.fmk failed to inhibit the nuclear apoptotic changes in such neurons.

It should be remembered that apoptosis is a complex process that can be triggered by several mechanism(s) at the same time. However, which caspase-independent mechanisms are responsible for apoptosis in the cultured slices remain to be investigated.

In the present study the survival of spinal cord slice from P7 mice was investigated. It was found that both neurons and glial cells died by apoptosis and that apoptosis occurs through a caspase-independent mechanism.

REFERENCES

1. Mouveroux, J. M., Lakke, E. A. & Marani, E. **(**2001). Lumbar spinal cord explants from neonatal rat display age-related decrease of outgrowth in culture. *Neurosci. Lett, 311*(2), 69-72.
2. Momeni, H. R. & Kanje, M. (2005). The calpain inhibitor VI prevents apoptosis of adult motor neurons. *Neuroreport, 16*(10), 1065-1068.
3. Lu, J., Ashwell, K. W. & Waite, P. (2000). Advances in secondary spinal cord injury: role of apoptosis. *Spine, 25*(14), 1859-1866.
4. Casha, S., Yu, W. R. & Fehlings, M. G. (2005). FAS deficiency reduces apoptosis, spares axons and improves

function after spinal cord injury. *Exp. Neurol*, *196*(2), 390-400.

5. Manabe, Y., Wang, J. M. & Murakami, T. (2001). Expressions of nitrotyrosine and TUNEL immunoreactivities in cultured rat spinal cord neurons after exposure to glutamate, nitric oxide, or peroxynitrite. *J. Neurosci. Res*, *65*(5), 371-377.
6. Momeni, H. R. & Kanje, M. (2006). Calpain inhibitors delay injury-induced apoptosis in adult mouse spinal cord motor neurons. *Neuroreport*, *17*(8), 761-765.
7. Darzynkiewicz, Z., Juan, G., Li, X., Gorczyca, W., Murakami, T. & Traganos, F. (1997). Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). *Cytometry*, *27*(1), 1-20.
8. Degterev, A., Boyce, M. & Yuan, J. (2003). A decade of caspases. *Oncogene*, *22*(53), 8543-8567.
9. Lockshin, R. A. & Zakeri, Z. (2002). Caspase-independent cell deaths. Curr. Opin. *Cell Biol*, *14*(6), 727-733.
10. Denizot, F. & Lang, R. (1986). Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods*, *89*(2), 271-277.
11. Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, *65*(1-2), 55-63.
12. Liu, Y., Peterson, D. A., Kimura, H. & Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem*, 69(2), 581-593.
13. Connelly, C. A., Chen, L. C & Colquhoun, S. D. (2000). Metabolic activity of cultured rat brainstem, hippocampal and spinal cord slices. *J. Neurosci. Methods*, *99*(1-2), 1-7.
14. Sgonc, R. & Gruber, J. Apoptosis detection: an overview. (1998). *Exp. Gerontol*, *33*(6), 525-533.
15. Compton, M. M. (1992). A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. *Cancer Metastasis Rev*, *11*(2), 105-119.
16. Martelli, A. M., Zweyer, M. & Ochs, R. L. (2001). Nuclear apoptotic changes: an overview. *J. Cell Biochem*, *82*(4), 634-646.
17. Cohen, G. M., Sun, X. M. & Fearnhead, H. (1994). Formation of large molecular weight fragments of DNA is a key committed step of apoptosis in thymocytes. *J. Immunol*, *153*(2), 507-516.
18. Cohen, G. M., Sun, X. M., Snowden, R. T., Ormerod, M. G. & Dinsdale, D. (1993). Identification of a transitional preapoptotic population of thymocytes. *J. Immunol*, *151*(2), 566-574.
19. Martelli, A. M., Bareggi, R., Bortul, R., Grill, V., Narducci, P. & Zweyer, M. (1997). The nuclear matrix and apoptosis. *Histochem Cell Biol*, *108*(1), 1-10.
20. Hendzel, M. J., Nishioka, W. K., Raymond, Y., Allis, C. D., Bazett-Jones, D. P. & Th'ng, J. P. (1998). Chromatin condensation is not associated with apoptosis. *J. Biol. Chem*, *273*(38), 24470-24478.
21. Taimen, P. & Kallajoki, M. (2003). NuMA and nuclear lamins behave differently in Fas-mediated apoptosis. *J. Cell Sci*, *116*(Pt 3), 571-583.
22. Yakovlev, A. G. & Faden, A. I. (2001). Caspase-dependent apoptotic pathways in CNS injury. *Mol. Neurobiol*, *24*(1-3):131-144.
23. Slee, E. A., Zhu, H., Chow, S. C., MacFarlane, M., Nicholson, D. W. & Cohen, G. M. (1996). Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone (Z-VAD.FMK) inhibits apoptosis by blocking the processing of CPP32. *Biochem J*. *315* (Pt 1), 21-24.
24. Susin, S. A., Lorenzo, H. K. & Zamzami, N. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, *397*(6718), 441-446.
25. Li, L.Y., Luo, X. & Wang, X. (2001). Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, *412*(6842), 95-99.