

ULTRAPROFOUND CEREBRAL HYPOTHERMIA AND BLOOD SUBSTITUTION WITH AN ACELLULAR SYNTHETIC SOLUTION MAINTAINS NEURONAL VIABILITY IN RAT HIPPOCAMPUS

Milos Ikonomic¹, Kevin M. Kelly², Teresa M. Hentosz³, Shou-Ren Shih⁴,
David M. Armstrong⁵, Michael J. Taylor^{4,6*}

Department of Psychiatry¹, University of Pittsburgh Medical Center, Pittsburgh, PA;
Departments of Neurology², Medicine³, and Surgery⁴, Allegheny General Hospital, Pittsburgh,
PA; MCP Hahnemann University², Philadelphia, PA; Lankenaw Institute for Medical
Research, Wynnewood, PA, and Department of Pathology, Anatomy, and Cell Biology,
Thomas Jefferson University⁵, Philadelphia, PA; Organ Recovery Systems, Inc., Suite 433,
MSC 1119, Port City Center, 701 East Bay Street, Charleston, SC 29403, USA^{4,6}

Summary

The acute effects of ultraprofound hypothermia and blood substitution (UHBS) on neuronal cell viability were examined in adult rat hippocampus, a brain region particularly vulnerable to ischemic cell death. UHBS was performed using either artificial cerebrospinal fluid (ACSF) or *Hypothermosol*, an "intracellular-type" hypothermic preservation solution. After the procedure, the hippocampus was sliced and tested for cellular viability using a combination of cellular fluorochromes that are markers for live cells (acridine orange) and dead cells (propidium iodide). UHBS with ACSF resulted in a variable degree of neuronal death within the hippocampal subfields CA1/CA3, and dentate granular layer and hilus (CA4). In contrast, UHBS with *Hypothermosol* consistently resulted in hippocampal slices with only mild neuronal death. Our results of preserved hippocampal neuronal viability with use of UHBS and *Hypothermosol* support the demonstrated central nervous system (CNS) protective effects of UHBS and *Hypothermosol* when used during prolonged cardiac arrest. The results of this study also suggest that UHBS and *Hypothermosol* may be useful in the preparation and maintenance of viable hippocampal tissue for physiological studies, especially those involving aged animals, which are particularly vulnerable to hypoxic-ischemic cellular injury.

Keywords: Aging, hippocampus, hypothermia, viability, ischemia, hypoxia

INTRODUCTION

The acute vulnerability of neuronal tissue to ischemic injury is well known and the role that UHBS can play in the context of clinical applications has been reviewed (11). Because of the brain's susceptibility to hypoxic-ischemic injury, basic research in neuroscience has a need to process brains with a minimum of injury caused by hypoxia and trauma. This is especially important in our own field of study that examines neural plasticity in aging brains, which can be exquisitely sensitive to hypoxic-ischemic injury (6).

The basic tenet of low temperature tissue preservation and protection is that cooling can significantly slow metabolic processes such that biological life can be extended during periods of interrupted blood supply and oxygen deprivation. Because the processes of tissue deterioration associated with ischemia and anoxia are mediated by chemical reactions, it has proved well founded to attempt to prevent or attenuate these changes by applying hypothermia. However, optimum cellular protection during hypothermia in organ preservation studies has been shown to be mediated by the specific design of the synthetic solutions used to perfuse and store the organs.

Collins and colleagues originally designed the first standard solution for clinical preservation of abdominal organs in 1969 (3). Since that time, the development of a variety of preservation solutions for organ storage has emphasized the need to optimize solution composition based on the particular characteristics of specific tissues. In recent years, the Collins solution has been superseded by a modified version called "EuroCollins", or by the "University of Wisconsin" solution (UW solution), which has emerged as the industry standard for kidney, liver, and pancreas preservation (8). Although certain isolated tissues and organs can be preserved adequately for days, the limits of tolerance of neurological tissue to hypothermic storage has not been established and currently there is no generally accepted preservation solution available for neuroprotection (11). In view of this, we have developed new hypothermic solutions that have been formulated as universal tissue preservation solutions to protect all tissues during whole-body blood washout and ultraprofound hypothermia. By design, the "*Hypothermosol*" solution contains components to a) minimize cell and tissue swelling, b) maintain appropriate ionic balance, c) avoid a state of acidosis, d) remove or prevent the formation of free radicals, and e) provide substrates for regeneration of high energy compounds and stimulate recovery upon rewarming and reperfusion. As such, *Hypothermosol* has been shown to be an excellent candidate solution to protect brain and other vital organs during prolonged cardiac arrest for bloodless surgery (7,10,11).

In view of current clinical concerns regarding potential neurological injury caused by transient hypoxic-ischemic events in patients undergoing a variety of complex surgical procedures, it is essential that techniques of CNS protection be more fully evaluated at the level of neuronal vulnerability. In order to achieve a better understanding of the degree of neuroprotection afforded by use of UHBS with *Hypothermosol*, we chose to study neuronal viability in the hippocampus, a brain region principally involved in memory functions and particularly vulnerable to hypoxic-ischemic injury. We examined neuronal viability with the use of neuropathological markers in order to broadly characterize physiological functioning after brief exposure to UHBS with *Hypothermosol*. Specifically, we developed a new live/dead cell-staining technique involving fluorochromes that have proven effective in other tissues (2) to examine selective neuronal vulnerability in rat hippocampal slices freshly prepared following UHBS with either *Hypothermosol* or ACSF. Our results are the first histological demonstration at a cellular level of the neuroprotective effects of UHBS with *Hypothermosol*. Furthermore, the technique described here proves to be an optimal and economical way to screen for the neuroprotective effects of synthetic hypothermic blood-substitution solutions in animal models. Finally, UHBS with *Hypothermosol* may be extremely useful in preparations for physiological studies of aged hippocampal tissue, which can be particularly vulnerable to hypoxic-ischemic cellular injury.

MATERIALS AND METHODS

Brain tissue was obtained from young adult Sprague-Dawley rats. Tissue was prepared by one of three methods: 1) UHBS with *Hypothermosol* (n=9); 2) UHBS with ACSF (n=8); and 3) decapitation and rapid removal (n=8). The ACSF contained (mM): 1.25 KH_2PO_4 , 26 NaHCO_3 , 128 NaCl , 3 KCl , 2 CaCl_2 , 2 MgCl_2 , and 10 glucose. This ACSF solution had a Mg^{2+} to Ca^{2+} ratio that was slightly higher than usual and a K^+ concentration that was slightly lower than usual to decrease neuronal hyperexcitability and promote viability of the hippocampal slice preparations (13). The formulation of the *Hypothermosol* maintenance solution used in this study has been published previously (10,11). *Hypothermosol* contained (mM): 7.5 KCl , 0.05 CaCl_2 , 5.0 MgCl_2 , 5.0 KHCO_3 , 10.0 KH_2PO_4 , 100.0 lactobionic acid, 25.0 HEPES buffer, 5.0 glucose, 20.0 sucrose, 20.0 mannitol, 100.0 NaOH , 20.0 KOH , 2.0 adenosine, 3.0 reduced glutathione, and 6% dextran-40 (40,000 MW).

Ultraprofound hypothermia and blood substitution (UHBS)

The methods of UHBS with *Hypothermosol* used in these experiments were adapted from those developed for a canine model (1,10). Briefly, rats were anesthetized and paralyzed by intraperitoneal injection of a chloral hydrate solution (3.5%; 1 cc/100g), atropine 0.02 mg/kg, and pancuronium 0.1 mg/100 g. Animals were put in an upright position on a fixation board and intubated endotracheally with a 2.5 cm blade laryngoscope and a 16-gauge catheter with guide-wire. A Harvard rodent respirator provided ventilation. A cranial burr hole was drilled and a temperature probe was inserted into the right olfactory bulb. Animals were prepared for cerebral perfusion with either *Hypothermosol* or ACSF by thoracotomy, cross-clamping of the descending aorta, scalpel-nicking of the right atrium to allow exsanguination, and puncture of the left ventricle by an 18 gauge hypodermic needle with ball tip to secure the needle tip within the ventricle. The perfusates were oxygenated and cooled to 4°C by a pediatric membrane oxygenator with low prime volume and a heat exchanger (Model M2, Jostra, Austin TX), and delivered to the heart tubing by a peristaltic roller pump (Rainin) through plastic tubing connected to the hypodermic needle. Using this technique, the perfused brains reached a low temperature of 8°C within approximately 3 min. While maintaining continuous perfusion with either *Hypothermosol* or ACSF, the brain was dissected from the cranium, and placed in ice-cold ACSF prior to slicing.

For comparison, some rats did not undergo ultraprofound cerebral hypothermia and blood substitution, but instead were decapitated following chloral hydrate anesthesia, and their brains were removed rapidly and placed into ice-cold ACSF prior to slicing. This latter procedure conforms to a technique commonly used in neuroscience research.

Brain Tissue Preparation and Hippocampal Slice Viability Assay

The excised brain was placed on an ice-cold glass dish where the rostral tip of the cerebrum and the entire cerebellum were removed with a razor blade. Slices were prepared following the procedure previously described for preparation of organotypic slices (9). Tissue slices of 400 μm were obtained with a Vibroslice VSL (WPI) vibratome. A horizontal cut was made slightly above the ventral surface of the brain where it was superglued to a block and placed in the Vibroslice chamber containing ice-cold ACSF. The brain was cut through the interhemispheric fissure into two halves and sliced horizontally in a caudo-rostral direction using a Gillette double-edged blade. Tissue slices were placed on mesh netting that was partially submerged in ACSF at 37°C in an interface chamber with 95% O_2 and 5% CO_2 for

~30 min to reestablish normal physiological functioning. Following revitalization, tissue viability was assessed using a method developed in our laboratories for applying the combined fluorochromes acridine orange (AO) and propidium iodide (PI) to brain slices. Tissue slices were put into 50 ml tubes containing AO (3 μ g/ml) and PI (10 mg/ml) in ACSF. The method results in green fluorescence of living cells and red fluorescence of dead cells. Slices were stained for 15 min, washed with ACSF several times, and fixed in 4% paraformaldehyde for 50 min. The slices were then processed for microscopic examination using a standard technique. Briefly, slices were washed several times in phosphate buffer (pH=7.4) and placed in 30% sucrose for cryoprotection in preparation for cryostat sectioning. After the slices sank in the 30% sucrose, they were removed and dissected into blocks containing the hippocampal formation. Hippocampal tissue slices were embedded in the Cryomatrix (Shandon) embedding resin, quickly frozen on dry ice, mounted onto a cryostat chuck, and cut into 15 μ m-thick sections. Sections were thaw-mounted on glass slides (Digene), examined, and photographed on a fluorescent Olympus BX-50 microscope equipped with appropriate filters (450nm excitation/520nm emission) and attached to an Imagemaster image analysis system.

Data Analysis

Because cells at the surface of a tissue slice sustain direct mechanical injury during slice preparation, the first five 15 μ m-thick cryostat sections (approximately 75 μ m of tissue) from the top face of a slice were excluded from the analysis. The most superficial of these injured tissue sections consistently showed widely distributed (indiscriminate) cell death and did not contain a complete hippocampal structure. The bottom face of the slice was not necessary to cut because it was glued to the chuck of the cryostat. Tissue sections were collected sequentially between these two end portions of a slice and displayed an intact cytoarchitecture of the hippocampal formation without evidence of non-selective (injury-related) cell death. Using this technique, we examined the middle, ~250 μ m-thick portion, of each 400 μ m-thick hippocampal slice producing approximately 15 sections/slice. Throughout these sections, the pattern of selective cell death remained consistent. After dissection of hippocampal slices, all tissue was kept in coded vials and remained coded throughout analysis. During the analysis of viability staining, two independent investigators remained blinded to experimental conditions. We focused our analysis on all principal hippocampal subfields, including the pyramidal cell layer of CA1 and CA3, the granule cell layer of the dentate gyrus, and neurons within the hippocampal hilus (CA4). Within these anatomically defined hippocampal areas, neuronal viability was assessed on a semi-quantitative scale by rating cell death as mild, moderate, or severe, based on predominance of green- (live) or red-fluorescing (dead) cells. Mean values of cell death with standard deviations were calculated.

RESULTS

The pattern of AO/PI fluorescence staining allowed us to examine the distribution of individual live (green fluorescence) and dead (red fluorescence) neurons within well-defined hippocampal subfields (Figure 1). The reliability of our staining technique was separately confirmed in selected hippocampal slices that were deliberately deprived of oxygen and glucose in order to promote cell death. These brain slices consistently showed a massive and indiscriminate cell death, classified as severe, throughout the hippocampus (data not illustrated). Brain slices from animals that underwent UHBS with ACSF perfusion or from

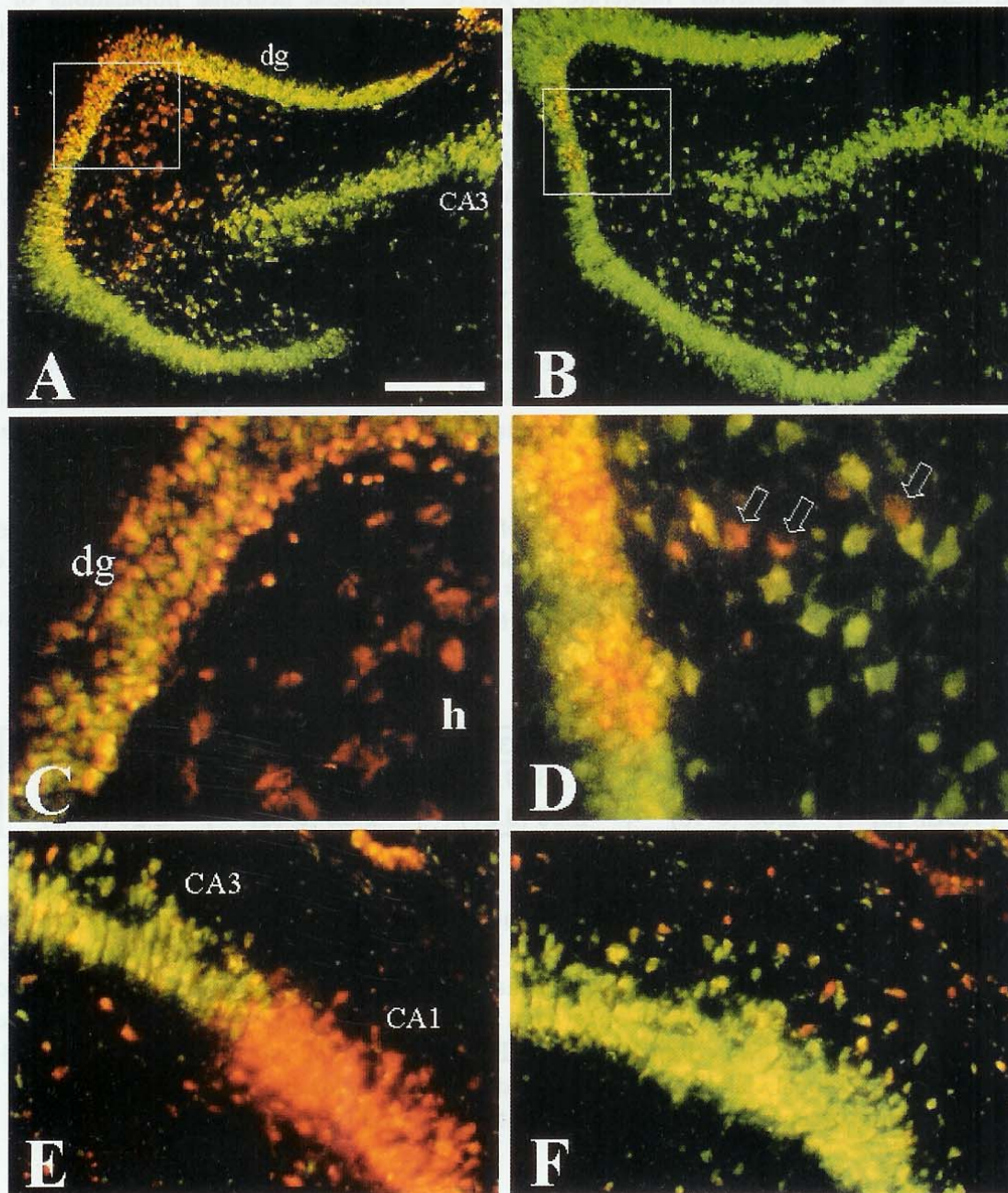


Figure 1. Fluorescence photomicrographs of hippocampal slices after brain perfusion with ACSF (A, C, E) or Hypothermosol (B, D, F) were stained with a combination of AO (green = live cells) and PI (red = dead cells) stains. ACSF-perfused tissue was characterized with selective cell death within the dentate gyrus, hilus, and the CA1 region (A, C, E, examples of severe cell death). Cell viability was consistently better in Hypothermosol-perfused hippocampal tissue. Although a small number of dead granular and hilar (arrows) neurons were present (B, D), Hypothermosol was generally associated with a higher degree of neuronal viability, particularly in the vulnerable CA1 region (F). C and D are enlarged details of boxed areas from A and B, respectively. Scale bar = 200 μ m (A and B); 50 μ m (C and D); 100 μ m (E and F).

those that underwent rapid decapitation and brain extraction, resulted in varying degrees of cell death that was generally moderate to severe. Principal neuronal populations of the hippocampus were particularly affected, including the more resistant areas (e.g., dentate gyrus). In this experimental group, substantial concentrations of dead cells could be found within some portions of the dentate granular cell layer and the CA4 hilar region (Figure 1, A and C). Dentate granule cells were particularly affected along the border with the hippocampal hilus. In addition, pyramidal cells of the CA1/CA3 subfields were also affected, often to a much greater extent than other hippocampal subfields. In some instances of severe CA1 cell death, a clear demarcation line toward less affected areas could be distinguished (see example in Figure 1, E). In contrast, UHBS with *Hypothermosol* consistently produced hippocampal slices with generally good viability of dentate granule cells (Figure 1, B and D) as well as the neurons within more vulnerable hippocampal regions, including the hilus and CA1 (Figure 1, D and F). Although the tissue that underwent UHBS with *Hypothermosol* also showed a mild degree of neuronal death in these slices, neuronal death appeared more sporadic within the hippocampus and much less frequent than that of the experimental group using ACSF (Table 1).

Table 1. Semi-quantitative analysis of neuronal viability in hippocampal tissue sections from HT- or ACSF-perfused animals. Values indicate the calculated mean cell death with standard deviation (SD): 1 = mild; 2 = moderate; 3 = severe. HT (Hypothermosol); ACSF (Artificial Cerebrospinal Fluid); CA1, CA3 (hippocampal CA subfields); H (hilus); DG (dentate gyrus).

Group (n)	CA1	CA3	H	DG
HT (9)	1.44 (0.18)	1.33 (0.17)	1.33 (0.17)	1.22 (0.15)
ACSF (8)	2.38 (0.18)	2.00 (0.27)	2.38 (0.18)	2.25 (0.16)

It should be noted, however, that the method of tissue preparation involved brief processing of all brain slices in cold ACSF, irrespective of the method of brain perfusion and extraction. Therefore, we cannot exclude the possibility that the mild degree of neuronal death detected in the Hypothermosol-perfused brains may have been induced during the subsequent stages of processing in cold ACSF. Regardless, UHBS with *Hypothermosol* generally resulted in notably better neuronal viability within all hippocampal subfields and particularly in those areas that were more susceptible to ischemic cell death (e.g., CA1).

DISCUSSION

The use of hypothermia as the principal means to suppress metabolism in a reversible way and thereby approach a state of "suspended animation" is the foundation of most of the effective methods for tissue and organ storage. Hypothermia is the basis of all useful methods of organ preservation and it has been proven to be most effectively applied by controlling the extracellular environment of cells directly and the intracellular environment indirectly during cold exposure. Preservation by cooling is achieved by striking a balance between the

beneficial and harmful effects of reducing temperature. Some of the basic principles that govern how this balance is best achieved are well understood whereas others are not. The main beneficial effect of cooling is the slowing of chemical reactions, including those of the ischemic cascade, thereby reducing the demand for oxygen and other substrates and conserving chemical energy. However, the details of the metabolic consequences of hypothermia are complex and poorly understood (12).

At a physical level, cooling has no useful effect on cell swelling or the redistribution of ions between the intracellular and the extracellular space, and rapid cooling may even be harmful. However, such changes can be restricted by appropriate design of the medium used to control the extracellular environment. This approach contrasts with a common practice in tissue banking to use “extracellular-type” solutions such as tissue culture media, as the base solution for preservation media. ACSF is also an example of an “extracellular-type” solution. However, there are good reasons why tissue culture media, which are designed to maintain cellular function at physiological temperatures, are inappropriate for optimum preservation at reduced temperatures. Maintenance of the ionic and hydraulic balance within tissues during hypothermia can be better controlled in media designed to physically restrict these temperature-induced imbalances. This is the fundamental basis for the design of synthetic acellular solutions used as blood substitutes under hypothermic conditions as described previously (10,11). The purpose of this study was therefore twofold: 1) to adopt a fluorescent viability assay for evaluation of neuronal cell death in areas of the brain known to be highly vulnerable to ischemia; and 2) to evaluate the comparative integrity of hippocampal subfields in rat brains perfused under hypothermic conditions with acellular solutions having either an “intracellular-type” formulation (*Hypothermosol*), compared with an extracellular-type” solution (ACSF).

We used a combination of live/dead cell markers (PI/AO) as a means to assess neuronal viability in hippocampus following UHBS. This assay was a modification of a previously described method for estimating the viability of isolated Langerhans islets from pancreas (2) and is comparable to the methodology developed for assessing cellular viability in brain using PI and fluorescein diacetate (4) and that used routinely in our laboratories to determine neuronal viability in cell toxicity experiments (5). We demonstrated that neuronal membrane integrity was substantially affected during UHBS with ACSF. In the hippocampus, a brain region particularly sensitive to hypoxic-ischemic cellular injury, this often resulted in widespread neuronal damage as characterized by the AO/PI staining. In contrast, we demonstrated that UHBS with *Hypothermosol* resulted in good overall hippocampal neuronal viability. Our results indicated that at the cellular level, *Hypothermosol* was neuroprotective during conditions of UHBS suggesting that this procedure may also be useful in ensuring viable neuronal tissue for physiological experiments in brains from aged animals since optimized techniques of brain tissue preparation are critical for maintaining cellular viability. Speed and gentleness of preparation are considered to be the most important factors in maintaining viability of the brain due to the vulnerability of neurons to injury from hypoxia and trauma. Preparation of brains from aged rats typically takes longer than that of younger animals due to the increased thickness of the skull and meninges from which the brain is removed. In addition, brains from aged animals are more susceptible to neuronal injury or death in routine preparation compared with younger animals. Brief periods of ischemia in aged neurons can cause critical oxygen deprivation that results in a rapid depletion of energy reserves, membrane depolarization, and biochemical events that cause pathological changes in neurons. During ischemia, calcium enters neurons through voltage- and ligand-gated ion

channels and accumulates in the cytosol. This intracellular calcium overload is known to be part of the cascade of events that can lead to irreversible cell damage and/or death. One method to minimize the deleterious effects of interrupted blood supply and oxygen deprivation to brain neurons is to subject the brain to hypothermic conditions which can be protective by slowing metabolic rates and decreasing energy use. Additionally, hypothermia also affects active ion transport and ion homeostasis, membrane fluidity and function, and the secretion of hormones and neurotoxins. In these ways, hypothermia can be beneficial although harmful effects can occur. Thus optimized neuronal protection and survival with hypothermia must balance the slowing of degradative processes and the accumulation of sublethal cellular injuries that can lead ultimately to cell death (11).

Future studies in our laboratories will employ the AO/PI assay in combination with various protein and mRNA markers to examine chronic effects of UHBS on neuronal viability. We will also attempt strict quantitation of individual live and dead cells within the hippocampus except for dentate granule neurons because of their small size and high packing density.

Acknowledgement: Ms. Laurie Machen and Mr. Mark Snyder provided excellent technical assistance throughout these studies. This work was supported in part by grants to KMK from The Nathan Shock Center of Excellence in the Basic Biology of Aging and the Institute on Aging, Allegheny University of the Health Sciences.

REFERENCES

1. Bailes JE, Leavitt ML, Teeple E, Maroon JC, Shih S, Marquardt M, Elrifai AM & Manack L (1991) *J Neurosurg* **74**, 781-788.
2. Bank HL (1988) *In Vitro Cellular & Developmental Biology* **24**, 266-273.
3. Collins GM, Bravo-Shugarman M and Terasaki PI (1969) *Lancet* **2**, 1219-1222.
4. Ikonovic S, Kharlamov E, Manev H, Ikonovic MD & Grayson DR (1997) *Neurochem International* **31**, 283-90.
5. Jones KH & Senft JA (1985) *J Histochem Cytochem* **33**, 77-9.
6. Kadar T, Dachir S, Schukitt-Hale B & Levy A (1998) *J Neural Transmission* **105**, 987-1004.
7. Simon D, Taylor MJ, Elrifai AM, Shih SR, Bailes JE, Davis D, Kluger Y, Diamond DL & Maroon JC (1995) *ASAIO J* **41**, M297-M300.
8. Southard JH & Belzer FO (1995) *Ann Rev Med* **46**: 235-247.
9. Stoppini L, Buchs PA & Muller D (1991) *J Neurosci Methods* **37**, 173-182.
10. Taylor MJ, Bailes JE, Elrifai AM, Shih SR, Teeple E, Leavitt MI, Baust JG & Maroon JC (1995) *Circulation* **91**, 431-444.
11. Taylor MJ, Elrifai AM & Bailes JE (1996) in *Advances in Low Temperature Biology*, (ed) PL Steponkus, JAI Press, London, pp1-64.
12. Taylor MJ (2000) in *Encyclopedia of Stress*, (ed) G Fink, Academic Press, New York, pp. 484-496.
13. Thibault O, Mazzanti ML, Blalock EM, Porter NM & Landfield PW (1994) *Neuroprotocols: Companion to Methods in Neurosciences* **4**, 177-181.

Accepted for publication 21/12/00