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Morphological and Functional Correlates

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FOREWORD

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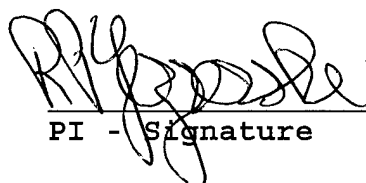
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ANNUAL REPORT (2000)

THERAPEUTIC HYPOTHERMIA FOLLOWING TRAUMATIC SPINAL INJURY

ROBERT P. YEZIERSKI, PH.D.

INTRODUCTION

The research carried out during the third year of funding focused on the effects of systemic hypothermia alone and in combination with the endogenous neuroprotective agent agmatine. In these studies we evaluated the effects of these treatments on morphological and behavioral outcome measures following traumatic spinal cord injury. We also evaluated the effects of post-traumatic hyperthermia on similar outcome measures. The discussion below describes the subject, purpose and scope of the research and the background of previous work related to: (a) the neuroprotective effects of hypothermia in models of CNS trauma and ischemia; (b) recent studies supporting the beneficial effects of modest cooling (1-5°C) in CNS injury; (c) the applicability of (a-b) to traumatic spinal cord injury (SCI); and (d) the neuroprotective effects of agmatine.

(a) Hypothermia and CNS Protection: The premiss that lowering CNS temperature protects against the detrimental effects of hypoxia and ischemia evolved in the 1950's. From a need to protect nervous tissue during vascular operations where circulation to the brain and/or spinal cord was interrupted hypothermia emerged as an adjunct to conventional therapeutic interventions. The protective influence of moderate and severe hypothermia was first demonstrated in experimental models of both spinal and cerebral ischemia (Beattie et al., 1953; Pontius et al., 1954, 1955; Marshall et al., 1956; Rosomoff, 1954, 1959), and its beneficial effects were concluded to be secondary to the lowering of cerebral metabolic demands. In early studies Rosomoff (1959) described a canine model of middle cerebral artery occlusion where hypothermic treatment (22-24°C for 1 hour) fifteen minutes after ischemia was protective against neurologic injury and death. Similarly, tolerance to interruption of cerebral circulation in dogs was dramatically increased (threefold) when body temperature was reduced to 23°-26°C (Marshall et al., 1956). Similar observations have been reported in primates subjected to prolonged periods (45-75 minutes) of cardiac arrest (Kopf et al., 1975). These observations led to the utilization of CNS cooling, albeit on a limited scale, as a form of treatment in pathological conditions such as stroke and spinal cord trauma.

Historically, methods for lowering CNS temperature to protect against the detrimental effects of hypoxia and ischemia were based on the observation that hypothermia reduces CNS metabolic activity and cerebral metabolic rate for oxygen. These effects decrease energy requirements of tissue and increase the period it can survive in an energy deficient state (Rosomoff and Holaday, 1954; Hägerdal et al., 1975, 1978; Kramer et al., 1968, Michenfelder and Theye, 1970). Hypothermia has also been shown to protect against loss of phosphocreatinine and the accumulation of lactate and NADH following cerebral hypoxia (Michenfelder et al., 1976; Hägerdal et al., 1978). In early studies hypothermia, for example, was found to decrease cerebral blood flow and oxygen consumption proportionally from 25°C to 35°C (Rosomoff and Holaday, 1954, Hägerdal et al., 1975). Systemic hypothermia was also shown to significantly lower the rate of cerebral ATP depletion following interruption of cerebral circulation (Michenfelder and Theye 1976, Kramer et al. 1968). Other beneficial actions of hypothermia include attenuation of edema and hemorrhage formation that occurs in SCI (Green et al., 1973) and the possible dialysation of toxins secondary to perfusing the cord with hypothermic solutions (Tator and Deecke, 1973). Recently, Tuzgen et al. (1998) reported that epidural cooling with chilled saline for 30 minutes following traumatic SCI significantly reduced secondary tissue damage due to the peroxidation of lipid membranes.

Neuroprotective Effects of Local Spinal Cord Cooling: Based on early studies involving brain injury, hypothermic techniques were modified to provide local cooling to the injured spinal cord. The technique was first successfully applied to experimental spinal cord injury by Albin et al. (1965) who perfused the traumatically injured spinal cord of dogs with cold isotonic saline (5°C) for 2.5 hours post-injury. As a result of this treatment there was a dramatic recovery of neurologic function compared to animals without this treatment. These investigators subsequently demonstrated beneficial effects of local spinal cord cooling (LSCC) in a similar SCI model in monkeys, even when the application of LSCC was delayed for four hours after injury (Albin et al., 1968). Following these observations, numerous experimental SCI studies ensued where animals were successfully treated with local hypothermia (Albin et al., 1965, 1968; Ducker and Hamit, 1969; Kelly et al., 1970; Black and Markowitz, 1971; Tator and Deecke, 1973; Campbell et al., 1973; Hansebout et al., 1975; Kuchner and Hansebout, 1976; Eidelberg et al., 1976; Wells and Hansebout, 1978).

In the assessment of beneficial effects of LSCC following SCI a variety of outcome measures have been used: (a) evoked potentials (Thienprasit et al., 1975); (b) degree of hemorrhage and edema formation (Green et al., 1973); (c) motor performance (Hansebout et al., 1975; Ducker et al., 1969; Thienprasit et al., 1975); and (d) histopathological analysis (Green et al., 1973). In many of these experiments LSCC was achieved via perfusion with a cold solution or an epidural heat exchanger and investigators aimed at achieving extremely low temperatures (in the neighborhood of 10-15°C below normal). In these studies spinal cord cooling following injury was found to be neuroprotective even if its application was delayed three to six hours post injury (Albin et al., 1968; Ducker and Hamit, 1969; Kelly et al., 1970; Thienprasit et al., 1975; Wells and Hansebout, 1978). Furthermore, the optimal duration of treatment was reported to be approximately four hours (Wells and Hansebout, 1978). Negative studies of LSCC following traumatic SCI have also been noted (Black and Markowitz, 1971; Howitt and Turnbull, 1972; Eidelberg et al., 1976). It should be emphasized, however, that differences in outcome may be secondary to differences in experimental design among different investigators including: (1) animal species; (2) anesthetic regime; (3) method of injury; (4) administration of other drugs; (5) opening the dura; and (6) techniques used to cool the cord. Additionally, spinal cord temperatures were not monitored in a similar fashion in all studies. Some investigators reported only epidural temperatures, and in large species the temperature gradient from epidural to the anterior column can be significant (Wells and Hansebout, 1978). In spite of the inconsistency of experimental design, lack of proper controls, and variability of injury models, most experimental data strongly support the beneficial effects of LSCC in experimental SCI.

The favorable results in animal experiments led to a limited number of cases where local cord cooling was used in human SCI patients (Selker, 1971; Meacham and McPherson, 1973; Koons et al., 1972; Negrin, 1975; Bricolo et al., 1976; Tator, 1979; Hansebout et al., 1984). The results, however, have been difficult to interpret for a variety of reasons: (1) most investigators report only a small number of cases; (2) controls have not been used in any series; (3) variability in level of injury; (4) results have been generally reported as the number of patients that improved or regained function as opposed to utilizing formal grading methods for measuring outcome; (5) the time interval from injury to application of cooling and duration of treatment have been highly variable; (6) a combination of different drug treatments (usually steroids) have been utilized in conjunction with LSCC; and (7) medical causes of spinal compression other than acute SCI, have been included in some studies. In spite of these complications Hansebout et al. (1984) in reviewing the application of this technique to humans concluded that the results were encouraging. The application of the technique, however, is fraught with technical and logistical difficulties not to mention the clinical challenge of performing a multilevel laminectomy on medically compromised patients (frequently with multiorgan trauma) while trying to minimize the time interval between injury and the application of cord cooling. These obstacles could be overcome if only modest systemic cooling was required in order to produce neuroprotective effects.

The largest patient series using hypothermia was reported by Meacham and McPherson (1973), in which 14 spinal injured-patients were treated with LSCC. Successful initiation of LSCC within 8 hours or less from the time of injury was achieved in all cases and the authors reported return of function in 7/14 patients. A major concern with this study, however, was a mortality rate of 29%, which the authors attributed to the frequency of respiratory complications that occur in cervical injuries. It is difficult to draw definitive conclusions regarding outcome from this study since controls were not utilized and the period of follow-up was not mentioned. Only two other studies contained more than a small number of SCI patients. The first was a series of 11 patients reported by Tator (1979) and the second was a series of 10 patients reported by Hansebout and colleagues (1984). Both studies found that LSCC provided functional recovery in 27% and 43% of patients respectively, which was considered to be higher than would be expected following conventional treatment.

(b) Mild Hypothermia In CNS Ischemia: The observation that modest hypothermia is neuroprotective in CNS ischemia was first demonstrated by Berntman et al. (1981) who observed that 1-5°C decreases in body temperature diminished the loss of ATP and phosphocreatinine, and lessened the degree of brain tissue acidosis following brain hypoxia. Recent studies in which actual brain temperature was monitored strongly suggest that a drastic lowering of CNS temperature is unnecessary to significantly reduce the degree of tissue damage occurring after brain ischemic injury. It has been documented that modest temperature changes (1-5°C) in models of brain ischemia and trauma can significantly alter the extent of neuronal injury, free radical activity and blood-brain barrier alterations (Busto et al., 1987; Dietrich et al., 1990; Dietrich et al., 1991, Globus et al., 1995). Furthermore, modest brain hypothermia reduces the release of neurotransmitters, such as glutamate, which can mediate secondary injury processes (Busto et al., 1989). These findings suggest that only modest changes in spinal cord temperature may be needed to lessen the extent of tissue injury following trauma. Consistent with this hypothesis are results showing systemic hypothermia lessens the neurological deterioration resulting from brain trauma (Bramlett et al., 1995) and improves neurological outcome following ischemia of the rat spinal cord (Robertson et al., 1986).

(c) The Application of Moderate Hypothermia in SCI: Based on the findings described above, it was hypothesized that mild changes in cord temperature could affect the extent of injury occurring in spinal cord trauma. The rationale for this hypothesis centered around the fact that: (1) local spinal cord cooling has been shown to be beneficial; (2) modest decreases in CNS temperature are effective in models of cerebral and spinal ischemia; and (3) modest decreases in brain temperature in models of CNS ischemia protect against processes which have been implicated in the pathophysiology of SCI, e.g. alterations in the blood-brain barrier, edema formation, production of leukotrienes, and release of neurotoxic substances such as glutamate and aspartate (Dempsey et al. 1987, Dietrich et al. 1990a,b, Busto et al., 1989).

The above observations raised the possibility that modest changes in spinal cord temperature may lessen the extent of tissue injury following trauma. Supportive of this idea Tator and Deecke (1973) reported normothermic perfusion to be as effective as hypothermic perfusion in experimental SCI of moderate severity. The actual temperature of the perfusing solution in these experiments was 36°C, which is mildly hypothermic. Low grade hypothermia is also known to spare ATP and phosphocreatinine concentrations, and decrease the magnitude of lactate accumulation after cerebral hypoxia (Berntman et al., 1981). Finally, systemic hypothermia has been shown to be neuroprotective in an experimental model of spinal ischemia (Robertson et al., 1986). Total body hypothermia in fact has been shown to be beneficial and is the standard of prophylaxis against ischemic SCI during aortic cross-clamping and cardiothoracic procedures involving controlled cardiac arrest. Its implementation in the treatment of brain injury in large trauma centers has been promising. Once parameters for optimal efficacy have been established in spinal injury, it seems logical that clinical trials in spinal cord hypothermia will offer similar rewards. *If hypothermia does provide important clinical benefits, it is of equal importance to the spinal injured patient to determine whether elevated systemic temperatures, as might be*

experienced during an episode of post traumatic fever, exacerbate the injury process and are detrimental to the recovery of function.

To investigate these hypotheses a preliminary study of modest hypothermia in a weight drop model of SCI in the rat was carried out (Martinez and Green, 1992). In this study female Sprague-Dawley rats (250-300g) were subjected to a 50 gram-centimeter (10 gram weight dropped 5cm) lesion at T8 under halothane-nitrous oxide anesthesia. Epidural temperature was maintained at 33°C in the first group of animals (n=3), and at 37°C in a second group (n=3). These temperatures were achieved by lowering systemic (rectal) temperature to 31-32°C in the hypothermic animals or raising systemic temperature between 38°C-39°C in normothermic animals. The epidural temperature in each group was maintained for four hours post trauma. Following injury, animals were kept under nitrous oxide anesthesia until the termination of the four hour treatment period. Three days post-injury animals were sacrificed and the spinal cords removed for histological examination. All animals remained completely paraplegic during this observation period. Morphological evaluation at the epicenter of lesion sites, however, revealed that the 38°C animals had significantly more hemorrhage and parenchymal damage than the 32°C animals (Martinez and Green, 1992).

In conclusion, local spinal cord cooling has been shown to be effective in the treatment of experimental SCI. Similar beneficial results have been reported in some clinical studies, but the number of patients is small and controls have not been utilized. In addition, the high mortality reported in some studies remains a major concern with its clinical application. Recent findings of the neuroprotective effects of modest hypothermia in brain ischemia, however, may be applicable to SCI and offer a treatment protocol with fewer complications. Indeed, preliminary observations suggest that modest temperature changes, such as can be produced via systemic hypothermia, can affect the degree of tissue injury following spinal trauma (Martinez and Green, 1992). The importance of such findings is that, compared to LSCC, systemic hypothermia provides a much simpler approach by which the cord can be "cooled" and thus obviates the need for acute surgical intervention. If effective, modest systemic hypothermia would provide an additional therapeutic approach that could be applied to the clinical treatment of acute SCI. Because of the effects on metabolic processes it is possible that mild hypothermia could extend the window of therapeutic opportunity for additional pharmacological interventions. Of equal importance hypothermia could be used in neurosurgical procedures of the spinal cord and vascular surgical procedures in which spinal cord perfusion may be compromised. Based on the above discussion it can therefore be concluded that there is sufficient justification in both the scientific and clinical literature for additional studies related to better defining the optimal parameters for the hypothermic treatment of the injured spinal cord.

(d) Neuroprotective Effects of Agmatine: The initial trauma induced by injury together with the complex cascade of secondary events following injury determines the degree of total tissue damage and the ultimate neurological outcome following SCI. These events include microvascular alterations, inflammatory processes, alterations of the biochemical environment, free radical formation, ischemia, and cell injuries (Anderson and Hall, 1993; Lipton and Rosenberg, 1994; Tator and Fehlings, 1991; Li et al., 1996). Presently, numerous agents are proposed to be neuroprotective against CNS injury (for recent reviews see McIntosh, 1993; Mattson and Scheff, 1994; Mocchetti and Wrathall, 1995). Steroids, neurotrophins, cytokines, and gangliosides have been demonstrated to promote neuronal survival or support neuronal growth in various *in vitro* systems (Mattson and Scheff, 1994; Blottner and Baumbarten, 1994; Olson et al. 1994.). Methylprednisolone improves neurological recovery when given early after human SCI (Bracken et al., 1990). Recently, neurotrophins and have also been utilized in several disease models: glial cell-derived neurotrophic factors (GDNF) in Parkinson's disease, nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF) in Alzheimer's disease, and insulin-like growth factor (IGF-1) in multiple sclerosis (Hefti, 1997) have been used as neuroprotective agents in an effort to combat the neurodegenerative etiology of these diseases. The neurotrophic factor basic fibroblast growth factor (bFGF) has also been reported to be neuroprotective in models of cerebral ischemia and traumatic brain injury (Koketsu et al., 1994; Fisher et al., 1995; Dietrich et al. 1996) and to protect neurons from axotomy-induced death (Peterson

et al., 1996). In a model of spinal cord compression, bFGF administered locally at the site of lesion was reported to improve hindlimb function in combination with methylprednisolone infusion (Baffour et al., 1995). Recently Teng et al. (1997) reported basic and acidic FGF to be neuroprotective for cholinergic neurons following contusion injury in the rat. In a study that was initiated during the first year of funding and completed during the second year we found that several neurotrophins and growth factors exhibited neuroprotective effects when delivered to the site of traumatic spinal cord injury (Lee et al., 1999).

In view of the long term desire to combine treatment modalities, e.g. hypothermia and pharmacological, we have continued to evaluate the neuroprotective properties of different pharmacological agents. Previously, we successfully demonstrated the neuroprotective effects of the decarboxylated arginine compound agmatine (Brewer et al., 1998) in an excitotoxic model of spinal cord injury. Since the discovery of agmatine a number of researchers have investigated the physiological role of agmatine in the CNS, e.g. where it is located, sites of action, and physiological role. Agmatine is a naturally occurring substance that is thought to be an endogenous neuroprotective transmitter. It is detectable in rat hippocampus, thalamus, hypothalamus, neocortex, ventral tegmental area and in the periaqueductal region of the midbrain (Otake et al., 1998). Agmatine is detectable in astrocytes (Regunathan et al., 1995) and neurons (Reis and Regunathan, 1998). Agmatine binds with high affinity to $\alpha 2$ adrenergic receptors (AR) (Pinthong et al., 1995) and to the putative imidazoline receptor (Li et al., 1994). Agmatine inhibits NOS (Auguet et al., 1995), nNOS and eNOS (Galea et al., 1996) and antagonizes the NMDA receptor (Yang and Reis, 1996, 1999). For these reasons we have been interested in the use of agmatine as a neuroprotective agent against the excitotoxic component of the secondary injury cascade following primary injury to the spinal cord. Supportive of the neuroprotective properties of agmatine are studies showing agmatine to have protective effects in rodent models of neurotoxic and ischemic brain injury (Gilad et al., 1995).

HYPOTHESES AND TECHNICAL OBJECTIVES

The experiments proposed in the original proposal were aimed at addressing a number of interrelated hypotheses focusing on defining the optimal hypothermic parameters required to produce neuroprotection and behavioral recovery in the injured spinal cord.

- 1. Hypothesis:** There is an interdependent relationship among systemic, epidural, and spinal cord temperatures that must be defined in order to determine the degree of systemic and/or epidural temperature required to produce neuroprotection in the injured spinal cord (Experiment 1, original proposal).
- 2. Hypothesis:** Due to the influence of temperature on a wide range of important homeostatic mechanisms necessary for maintaining the structural and functional integrity of spinal tissue, it is proposed that increases in systemic or site of injury temperatures (*hyperthermia*) will accelerate the injury process and compromise behavioral recovery, whereas reducing the temperature (*hypothermia*) will result in neuroprotection and enhanced behavioral recovery (Experiment 2, original proposal).
- 3. Hypothesis:** There is an optimum time (post-injury) when post traumatic hypothermia of injured tissue produces the greatest benefit and is most effective in reducing behavioral deficits and morphological damage (Experiment 3, original proposal).
- 4. Hypothesis:** There is an optimum duration of hypothermic treatment which results in the greatest benefit to neurologic outcome (Experiment 4, original proposal).
- 5. Hypothesis:** Combining hypothermia with a pharmacological treatment will result in an additive or synergistic effect on morphological and behavioral outcome measures (Experiment 5, original proposal).

MILITARY BENEFIT OF PROPOSED STUDIES: If modest decreases in spinal cord temperature, achieved by systemic hypothermia or local cord cooling, are capable of reducing the degree of tissue damage following SCI, such a finding would have a significant impact on current protocols used in the treatment of acute spinal cord injury. The military importance of such a finding relates to the fact that in combat situations there are a limited number of options available for the treatment of spinal injured soldiers. If cooling the spinal cord is a viable treatment option the ability to cool the cord using transcutaneous or systemic hypothermia would provide a much simpler approach in order to gain the benefits of hypothermic neuroprotection. If effective, modest systemic hypothermia would provide a therapeutic treatment which could be applied to the human condition at times when surgical intervention is logistically difficult or when multiple injuries make other modes of treatment difficult to implement without severely compromising the survival of the patient. *Conversely, it is important to determine the detrimental effects of elevated systemic and cord temperatures in exacerbating the injury process. Since fever secondary to pulmonary compromise and stressed immune function is a common finding in traumatically injured patients, it is important to establish the clinical consequences of modest elevations in systemic temperature (in terms of augmented tissue damage).* This information is especially important in the design of treatment protocols for soldiers that develop febrile conditions post injury.

STATEMENT OF WORK: THIRD YEAR

During the third year of funding the primary goals were to complete the evaluation of the effects of hyperthermia and hypothermia on behavioral and morphological outcome measures following traumatic spinal cord injury (SCI). Additionally we wanted to continue our evaluation of the effects of agmatine and agmatine plus hypothermia on these outcome measures. These studies were intended to extend those carried out during the second year in which it was shown that moderate hypothermia delivered for a period of four hours was neuroprotective following moderate, but not severe, traumatic SCI. Although local spinal cord cooling has been attempted as a form of treatment in experimental and human SCI, most studies have focused on temperature shifts in the range of 15-20°C. Because of the technical difficulties required to achieve these conditions the application of hypothermia as a therapeutic intervention in SCI has been difficult to implement. Recent experimental data, however, suggests that modest changes (1-5°C) in central nervous system (CNS) temperature may positively influence outcome following CNS injury. The specific aim for the past year was, therefore, intended to utilize a clinically relevant model of contusive spinal injury, i.e. weight drop, and evaluate the effects of systemic hypothermic treatment on morphological and behavioral endpoints following injury. Since we would like to ultimately evaluate the neuroprotective effects of combination therapies, e.g. hypothermia with pharmacological treatments, we also initiated a study to evaluate the neuroprotective effects of the endogenous neuroprotective agent agmatine, both alone and in combination with hypothermia. As described above agmatine has been shown to have neuroprotective properties in models of traumatic and ischemic head injury and excitotoxic spinal cord injury.

1. Based on observations that slight decreases in brain temperature can significantly improve neurologic outcome following ischemic or traumatic brain injury, it was hypothesized that modest decreases (1-5°C) in spinal cord temperature would provide neuroprotection and enhanced behavioral recovery following traumatic spinal injury. In experiments carried out during the first year, we determined that lowering the epidural temperature to 32°C for a period of four hours post injury produced significant neuroprotection within the injured cord. During the second year we extended this research by evaluating the effects of hypothermia on functional outcome. In the third year we also evaluated the detrimental effects of systemic hyperthermia (39.5-40.0°C) on morphological and behavioral outcome measures.

2. Determine the effects of combining the most beneficial hypothermic regime with a protocol of pharmacological treatment. In these experiments we used the temperature of 32°C, the application time of 30 minutes post injury, and a duration of four hours in combination with systemically delivered agmatine (100mg/kg) and evaluated behavioral and morphological endpoints.

EXPERIMENTAL DESIGN AND METHODS

General Methods

Experimental models of spinal trauma: Although many models have been developed for the production of spinal cord injuries in animals, no single model can perfectly mimic the human condition. Several models, such as the weight drop or Allen (1911) technique and the aneurysm clip compression technique, have been well characterized and documented to create graded, reproducible, spinal cord injuries in rats (Rivlin and Tator, 1978, Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). The weight drop method involves dropping a known weight (usually 10g in rodents) a selected distance. As the height of the drop is increased, more severe injuries are produced. In spite of requiring a laminectomy and several reports of it yielding variable neuropathologic changes, the weight drop model remains an accepted standard technique which closely mimics the biomechanics of the human injury and produces injuries which are morphologically similar to those seen in humans (Jellinger, 1976). Furthermore, it has been shown that under careful control of experimental variables, this model will yield reproducible graded injuries (Gale et al., 1985, Wrathall et al., 1985, Noble et al., 1985). In studies being carried out in the Research Plan injuries are produced using a weight drop device obtained from Dr. Wise Young at New York University. This device has been used to produce a standard injury in a multi-center study designed to evaluate the clinical efficacy of drug actions in the treatment of acute spinal cord injury. The injury model to be used is therefore one that has widespread use in the field of spinal cord injury. This feature offers significant advantages, i.e. standardization, when comparing results obtained with different putative therapeutic interventions, e.g. hypothermia versus drug treatments.

Surgical Preparation: Anesthetized adult (250-275g) female Sprague-Dawley rats had the ventral aspect of their neck and back shaved and scrubbed with betadine solution. Level of anesthesia is assessed by monitoring arterial pressure, corneal reflex, and hindlimb withdrawal to noxious stimuli. Using aseptic techniques, PE catheters are placed in the external carotid artery for blood pressure and heart rate monitoring, and in the external jugular vein for fluid and drug administration. Rats are orotracheally intubated with a PE 220 catheter, paralyzed with pancromonium bromide (0.6 mg i.v. followed by a 0.1 mg/kg/hr infusion), and artificially ventilated (Ugo Basile rodent respirator). An anesthetic regime of Halothane, oxygen, and air (see below) is adjusted to maintain physiologically normal levels of pO₂ and pCO₂. Arterial blood gases are measured every hour with a blood gas analyzer (Radiometer ABL330; 75µl samples). Animals are paralyzed in order to: (1) fully control an animal's respiration and eliminate hypercarbic and hypoxemic effects of anesthetic agents; and (2) ensure physiologically normal blood gases and therefore mimic conditions as they occur in humans at the time of injury.

Contusive spinal cord injury: For producing traumatic spinal injury, a T-8 laminectomy is performed and animals positioned in the weight-drop apparatus as described by Noble and Wrathall (1987). The severity of injury is varied by adjusting the height of the weight drop (10g weight) as follows: mild injuries (5cm), moderate (12.5cm), and severe (25.0cm). Modifications to the parameters (weight and height) used for production of injuries were made based on evaluations of injuries. At the conclusion of surgery the incision is closed in layers, and catheters removed. Post-operatively, animals are housed in cages containing soft bedding and treated with cefazolin (40 mg) i.m. twice a day for 5 days. Water bottles were placed sufficiently low to allow access to water. Food was placed inside the cage until the rats are capable of reaching the standard placement in the cage top. Injured animals were checked daily and bladders palpated at least twice daily and emptied as required until they gain reflex voiding. Body weight was monitored weekly and records kept of all animal care. Antibiotics were administered to animals exhibiting signs of urinary tract infection. Veterinary consultation was obtained for animals demonstrating discomfort or autonomy following injury. In our experience, autonomy was rare in animals with T8 spinal cord injuries. Inclusion criteria for animals to be used in the study consisted of: (a) paraplegia post weight drop; (b) spinal cord hematoma; (c) acceptable weight drop (compression, impact velocity, impact height); and (c) acceptable physiological parameters (blood pressure, pCO₂, blood pH).

Measurement and variation of temperatures: Systemic temperature is controlled with a temperature circulator connected to a cooling or warming blanket (Lauda RM6-6 unit which is accurate to 0.1 °C). A flexible thermistor probe (Physitemp IT-21, 410µm diameter) is inserted in the rectum to monitor systemic temperature, and a second thermistor placed laterally at the site of laminectomy in the epidural space to monitor epidural temperature.

Anesthetic Regime: The anesthetic regime consisted of Halothane (0.5-5.0%), nitrous oxide (20%) and oxygen (20%) in order to produce physiologically normal levels of pO₂ and pCO₂. The rationale for using this anesthetic combination is: (1) inhalational anesthetics provide a much easier induction, a more uniform level of anesthesia, and a more prompt recovery than injectable anesthetics such as barbiturates or ketamine; and (2) since halothane is commonly used in all studies carried out in The Miami Project, this anesthetic enables comparisons of results between studies.

During the first year of the funding period it should be noted that we made a modification in the anesthetic mixture given during surgery. The anesthetic regime consisted of isoflurane (0.5-5.0%), nitrous oxide (20%) and oxygen (20%) in order to produce physiologically normal levels of pO₂ and pCO₂. The change was made as it was noted that we experienced an unusually high mortality rate which was attributed to cardiovascular complications of halothane. In the second year the switch was made back to halothane after a thorough overhall of our anesthetic and respiratory equipment was carried out. During the second year we did not experience any problems with regard to animal survival using halothane as an anesthetic.

Agmatine Administration: In experiments evaluating the effects of agmatine the drug was administered (I.P.) within 15 minutes after injury. After the initial injection, daily injections were given (at the same time each day) for a period of 14 days. Agmatine was dissolved in normal saline and administered at a dose of 100mg/kg. This injection protocol was used in experiments when agmatine was evaluated alone or in combination with hypothermia.

In all experiments each group of animals being evaluated consisted of a minimum of 5 animals. Statistical comparisons were carried out using analysis of variance (ANOVA). The volume of tissue damage, are presented as mean + standard error, and p-values of <0.05 were considered significant (see Appendix).

Histopathology of Experimental SCI: The morphological changes associated with experimental SCI have been documented in a variety of species (Allen, 1914, Ducker et al., 1971, Bresnahan et al., 1976, Balentine 1978a, 1978b, Noble and Wrathall, 1985). Hours following traumatic SCI hemorrhagic changes progress centrifugally and injured areas coalesce to form an area of hemorrhagic necrosis that extends along the longitudinal axis of the cord in a spindle shaped form (McVeigh, 1923, Ducker et al., 1971, Balentine, 1978a). The acute damage is located more centrally and, depending on the severity of the injury, it may progress to involve the adjacent white matter (Ducker et al., 1971). White matter changes begin in the areas adjacent to the gray matter and spread outward in a centrifugal fashion (Bresnahan et al, 1976, Bresnahan, 1978). By the end of the first week postinjury, demyelination and cystic degeneration of necrotic areas becomes evident, particularly in more severe injuries (Ducker et al., 1971, Blight, 1985). By four weeks, the cystic cavity is better defined and the surviving white matter displays demyelination and microcysts (Wagner et al., 1978, Noble and Wrathall, 1985). At four months the cyst is surrounded by astrocytic gliosis and the region of injury shows thickening of the dura mater. An increased cellularity of the leptomeninges is apparent, especially in the more severe injuries (Wagner et al., 1978). Although the morphological analysis to be used in the present study was not extended four months, many of the same analytical protocols alluded to above will be used (see below).

Histological evaluation: At the termination of experiments, animals are deeply anesthetized with sodium pentobarbital and perfused transcardially with a solution of 4% paraformaldehyde and 3.6% glutaraldehyde in 0.1 M Sorensen's phosphate buffer. Injured cord segments, along with surrounding normal cord were removed.

(a) Data Analysis: An important aspect of all experiments is the quantification of results. In order to establish

meaningful relationships among different treatment groups, it was imperative to quantify the amount of tissue damage for animals undergoing different treatments. To this end, transverse or horizontal sections were examined with light microscopy and preliminary reconstructions of the area of tissue damage, i.e. neuronal loss, axonal injury, were made with the aid of an overhead projector and camera lucida (using 1X or 4X objectives). This analysis was carried out by an individual "blinded" to the experimental design for tissue being analyzed. The area of maximal gray and white matter damage at the epicenter of injury sites was evaluated using computer aided image analysis (Image I, Universal Imaging Corp.). This technique has been used successfully in studies to quantify the amount of gray and white matter damage resulting from weight drop injury of the rat cord. This method involves the use of 20-30 longitudinal (horizontal) serial sections. In horizontal section the rostrocaudal boundaries of tissue damage can be found easily by evaluating the presence or absence of inflammatory cells, necrotic tissue, and macrophages. Using a low power (1X) objective camera lucida drawings are made of the gray matter of sections from the tissue block. Each area is then traced onto a digitizing tablet (Summagraphics) interfaced to a MicroVAX computer system, which computes areas at each horizontal level. The total necrotic area is derived by means of numerical integration of sequential areas. Based on results obtained during the past two years this method has provided an effective approach to quantitatively describing the region of tissue damage resulting from weight drop injury in the rat.

Behavioral Outcome: A number of methods have been devised for the assessment of residual neurological function following experimental SCI. The most widely utilized are modifications of Tarlov's score, which is an assessment of spontaneous locomotion (Tarlov, 1957), and the inclined plane score described by Rivlin and Tator (1977). Several other tests have been utilized, such as the response to paw pinch and reflex righting (Gale et al, 1985). Recently tests of sensory function including mechanical and thermal sensitivity (Hargreaves et al., 1988; Bennett and Xie, 1988; Hama and Sagen, 1993) have provided testing paradigms designed to evaluate the effects of injury on sensory systems. Although none of the above mentioned tests specifically address the integrity of individual spinal pathways, they do provide an index of the severity of injury and of an animal's overall neurological state. In our evaluation of behavioral recovery following hypothermia and/or agmatine we used the Basso-Beattie-Bresnahan (BBB) locomotor rating scale (Basso et al., 1995). This scale is a multiple function test of locomotor outcome which provides an efficient, expanded and unambiguous locomotor rating (Basso et al., 1995). In the original proposal it was suggested that in addition to the BBB test that the following tests would also be used to assess neurological function: (1) inclined plane score - measuring the steepest angle a rat is able to maintain its position for at least five seconds; rats are tested with their heads facing right, left, up and down; (2) righting reflex - a measure of a rat's ability to return to the upright position after being placed flat on its back; in addition to hindlimb function this test also assesses trunk muscle function; (3) response to paw pinch - rats are scored on their response to paw pinch on a 0 (no response) to 4 (normal response) scale (a variant of this test using calibrated von Frey filaments to evaluate responses to mechanical stimuli can also be used); and (4) thermal paw flick - this test measures the response of an individual paw to varying intensities of thermal stimuli. Due to time constraints and because of the thorough evaluation achieved with the BBB, it was decided that tests 1-4 (above) would not be used.

Prior to using the BBB test pre-injury training sessions (two sessions for each rat) were carried out to familiarize the animals with the environment of the room in which they were tested and to get them used to being handled. Behavioral testing was carried out beginning on day 2 post-injury and was performed in a blinded fashion on days 2,5,9,12,16,19,23,26,30,33,37,40,44 post injury.

EXPERIMENTS CARRIED OUT DURING THE THIRD YEAR OF FUNDING**Experiment 1**

Specific Aim: *Evaluate the effects of mild hypothermia on locomotor function following traumatic spinal cord injury.* The objective of these experiments was to study the effects of post traumatic hypothermia on behavioral outcome measures in animals subjected to mild traumatic SCI. This series of experiments combined results described in Experiments 1-4 of the original proposal.

Rational: During the first year it was demonstrated that mild hypothermia delivered for a period of 4 hours thirty minutes after injury resulted in significant neuroprotection following traumatic SCI. An important question related to this effect is whether there is any behavioral significance attached to this neuroprotective effect.

DETAILED RESULTS OF THIS STUDY ARE DESCRIBED IN THE PAPER BY YU ET AL. WHICH IS NOW IN PRESS IN THE *JOURNAL OF NEUROSURGERY* (SEE APPENDIX).

Experiment 2

Specific Aim: *Evaluate the effects of the systemic administration of agmatine on locomotor function following traumatic spinal cord injury.* The objective of this series of experiments was to study the effects of agmatine on morphological and behavioral outcome measures in animals undergoing traumatic SCI. This series of experiments is related to the objectives in Experiment 5 of the original proposal.

Rationale: Previously it has been shown that agmatine is neuroprotective in models of CNS injury (trauma and ischemia). Our own work has shown that agmatine administered systemically or intraspinally also produces significant neuroprotective effects against excitotoxic tissue damage produced by intraspinal injections of quisqualic acid. Considering the fact that agmatine is an NMDA antagonist and an inhibitor of NOS, we wanted to determine if agmatine could produce neuroprotective and/or behavioral effects following traumatic SCI. Based on our long term goals of combining therapeutic strategies with hypothermia it was also thought that this evaluation was an important first step towards accomplishing this goal with a substance that could be easily administered., i.e. systemically.

THE RESULTS OF THIS STUDY ARE DESCRIBED IN THE MANUSCRIPT BY YU ET AL. WHICH IS NOW IN REVIEW IN THE JOURNAL *SCIENCE* (SEE APPENDIX).

Experiment 3

Specific Aim: *Evaluate the effects of mild hyperthermia on locomotor function following traumatic spinal cord injury.* The objective of these experiments was to evaluate the effects of post traumatic hyperthermia (39.5-40.0°C) on behavioral outcome measures in animals subjected to traumatic SCI. This series of experiments combines the results from Experiments 1-4 of the original proposal and addresses the objective in Experiment 5.

Rationale: Considering the positive effects of hypothermia on behavioral outcome measures following traumatic SCI, the hypothesis was proposed that the opposite effect, i.e. increasing the temperature, would have a detrimental effect on behavioral and morphological outcome measures.

Protocol: A protocol combining those described in Experiments 1 was used in this study.

Results: The results have shown that mild hyperthermia results in significant deterioration of spinal tissue compared to control animals. In our analysis of differences in the tissue damage in these two groups of animals we have observed a 10-15% difference in the overall area of damage between these two groups of animals. Additionally, there were significant effects at early and late time points for animals in the hyperthermia group compared to the BBB scores of animals in the normothermic group. This conclusion is based on the significant difference in the final BBB scores obtained in the hyperthermia vs. normothermia. Although the results of this comparison show the disadvantage of hyperthermia it will be important to extend these results to determine if hyperthermia plus pharmacotherapy, e.g. agmatine, reduces these effects. To test this hypothesis one would need to administer agmatine at different time points during the period of hyperthermia and determine if similar results to agmatine administered at 30 minutes post injury would be obtained.

CONCLUSIONS AND FUTURE DIRECTIONS

During the third year of funding we determined that mild hypothermia (32°C) delivered within thirty minutes of a moderate injury for a period of four hours produces significant differences in locomotor scores when compared to normothermic animals. This result combines findings from the first and second years in which we determined the most effective duration of treatment, onset time, and injury severity required to produce neuroprotective effects of hypothermia. In a parallel series of experiments not directly related to the research plan of the hypothermia project we also determined that administration of the NMDA antagonist and NOS inhibitor agmatine also produces significant neuroprotective effects against excitotoxic injury resulting from the intraspinal injection of quisqualic acid. These effects were achieved with either intraspinal or systemic administration of agmatine. The encouraging results of this study led to a series of experiments closer to the hypothermia research plan an included an evaluation of agmatine effects against traumatic SCI. In these experiments we determined that the systemic administration of this drug produced significant behavioral effects. Because these effects could be achieved with systemic administration of the drug, unlike the effects of cytokines and growth factors evaluated in the first year which were obtained with intraspinal injection and our desire to combine hypothermia with a pharmacological intervention , the results with agmatine led us to the evaluation of combining hypothermia with the systemic administration of agmatine. Unfortunately, the combination of hypothermia and agmatine did not result in an additive or synergistic effect on behavioral outcome suggesting that hypothermia should be combined with an intervention directed at another component of the secondary injury cascade.

In the fourth year of funding (no cost extension year) there are three objectives that we would like to accomplish:

(a) Evaluate the effects of 8 hours of hypothermia commencing within 30 minutes of injury. To date our research has shown that mild hypothermia delivered for a period of 4 hours produces significant behavioral effects. To determine if a longer duration of hypothermic treatment is capable of producing an even greater morphological and behavioral effect we believe it is possible to carry out a study using the 8 hour time frame. Because of the need to keep animals anesthetized throughout the hypothermic period, it is felt that this is the upper limit that can be realistically evaluated . This study will address the objective described in Experiment 4 of the original proposal.

(b) One of the goals of the original research plan was to evaluate the effects of hyperthermia on morphological and behavioral outcome measures. To complete our evaluation of the effects of temperature on recovery from spinal cord injury, i.e. hypothermia and normothermia, we would like to continue our evaluation of the effects of hyperthermia on our morphological and behavioral outcome measures. To date we have demonstrated the detrimental effects of hyperthermia and now we would like to evaluate whether these effects can be reversed by pharmacological intervention This study will address an objective described in Experiment 2 of the original proposal.

(c) Recently it was shown that systemic administration of the anti-inflammatory cytokine IL-10 produces significant morphological and behavioral effects on a moderate injury of the spinal cord. Furthermore, no effects were found on more severe injury. In the first year of the funding period we determined that mild hypothermia by itself also did not produce any significant effects on a severe injury. Therefore, no interventions have been developed that result in beneficial effects on the most severe of injuries. Because of the clinical relevance of the severe injury, we believe it would be important to evaluate the effects of hypothermia and IL-10 on the severe injury. If successful this combination of interventions would offer a therapeutic strategy for any severity of spinal cord injury. We believe that this combination of effects is a reasonable combination, due to the fact that IL-10 produces its effect by effecting the inflammatory component of injury and because IL-10 is administered systemically. We will also evaluate the effects of the combination of these interventions on the moderate injury. This study combines results from Experiments 1-4 and addresses the objective described in Experiment 5 of the original proposal.

(d) The final objective is to try to determine if there are physiological correlates to the improved behavioral effects demonstrated during the past year. To date we have demonstrated the therapeutic efficacy of mild systemic hypothermia on behavioral and morphological outcome measures. We would now like to complement these studies by evaluating the mechanism of this behavioral recovery in physiological studies focusing on sensory and motor circuits in the spinal cord. These studies address objectives outlined in Experiments 1-4 of the original proposal.

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APPENDICES

ABSTRACTS:

1. Yu, CG, Marcillo, AE, Dietrich, WD, Fairbanks, CA, Wilcox, GL, Yeziarski, RP **Beneficial effects of hypothermia and/or agmatine on locomotor outcome following contusion spinal cord injury.** *Neurosci. Abstr.*, 1999.

PAPERS:

1. Yu, CG, Marcillo, AE, Dietrich, WD, Fairbanks, CA, Wilcox, GL, Yeziarski, RP **Agmatine improves locomotor function and reduces tissue damage follwoing traumatic spinal cord injury in rats.** *Science* (in review).

2. Yu, CG, Jimenez, O, Marcillo, AE, Weider, B., Bangerter, K, Dietrich, WD, Castro, S, Yeziarski, RP **Beneficial effects of modest systemic hypothermia on locomotor outcome and histopathological damage following contusion spinal cord injury in rats.** *J. Neurosurg.* (In Press).

3. Yu, CG, Jimenez, O, Marcillo, AE, Weider, B., Bangerter, K, Dietrich, WD, Castro, S, Yeziarski, RP **Detrimental effects of modest systemic hyperthermia on locomotor outcome and histopathological damage following contusion spinal cord injury in rats.** *Neurosurgery* (In Preparation).

**Agmatine Improves Locomotor Function and
Reduces Tissue Damage
Following Traumatic Spinal Cord Injury In Rats**

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ABSTRACT

Clinically effective drug treatments for spinal cord injury (SCI) remain unavailable. Agmatine, a N-methyl-D-aspartate (NMDA) receptor antagonist and inhibitor of nitric oxide synthase (NOS), is an endogenous neuromodulator expressed in brain and spinal cord. Evidence is presented that agmatine significantly improves locomotor function and reduces tissue damage following traumatic SCI in rats. The therapeutic targets of agmatine (NMDA receptor and NOS) have been shown to be critically linked to the pathophysiological sequella of SCI. These data suggest the importance of future strategies encompassing the use of single drugs with multiple therapeutic targets for the treatment of acute SCI.

Traumatic spinal cord injury (SCI) has been estimated to occur in 3 per 100,000 people resulting in approximately 15,000 new cases per year (1, 2). At present the recommended treatment for acute SCI is the glucocorticosteroid methylprednisolone (MP) that is believed to have multiple actions including stabilization of membranes, reduction of edema, and an inhibitory effect on lipid peroxidation. Unfortunately, many patients with spinal injury remain physically impaired as a result of their injury (2, 3). For this reason continued efforts are needed to develop and test novel treatment strategies directed at specific components of the complex injury cascade associated with SCI.

Experimental strategies designed to limit the extent of tissue damage following SCI have relied, in part, on the targeting of NMDA receptors (4), the synthetic pathway for nitric oxide (5), or inflammation (6). Injury-induced elevations in excitatory amino acids, including glutamate, and the induction of nitric oxide synthase (NOS) by NMDA receptor activation have been implicated as important steps in secondary injury following brain and spinal cord injury (7, 8, 9). Consistent with this are the neuroprotective properties of MK-801 (28, 29) and putative inhibitors of different NOS isoforms in brain and spinal cord injury, including aminoguanidine that inhibits iNOS (7). Although single drug pharmacotherapy is an important strategy for the treatment of acute SCI, the temporal profile of various post-injury chemical cascades may require combination therapy with multiple drugs administered at different time points. Future treatments for acute SCI may therefore sequentially target specific components of these post-injury cascades. An alternative to this strategy is the use of a single drug with multiple

therapeutic targets. The present study supports this latter strategy by showing that agmatine, a NMDA receptor antagonist (15) and inhibitor of NOS (17) produces a significant restoration of locomotor function and reduction of tissue damage following traumatic SCI in the rat.

The pathological sequella associated with traumatic SCI includes white and gray matter damage resulting from the primary injury and a progressive secondary injury that begins with the elevation of excitatory amino acids (9). Importantly, NMDAR and NOS are known to play important roles in the progression of secondary injury initiated by brain and SCI (4,10-12). Agmatine (decarboxylated arginine) is an endogenous neuromodulator expressed in brain (13) and spinal cord (14) with both NMDAR antagonist (14, 15) and NOS inhibitor activities (16, 17). Furthermore, agmatine does not impair motor function or have sedative side effects at either the effective dose range or four times the effective dose required to block thermal hyperalgesia in mice (14). Thus, the low toxicity profile combined with the putative sites of action makes agmatine an attractive therapeutic agent (18).

To evaluate the effects of agmatine on locomotor function and pathological damage caused by SCI, a clinically relevant contusion model of spinal cord injury was used (NYU impactor) (19-21). Rats were randomized to saline-treated and agmatine-treated groups (22). Agmatine was administered i.p. 30 minutes after SCI at a dose of 100mg/kg/day for 14 days. The dose of agmatine (100mg/kg) was based on results of a previous dose-response determination using an excitotoxic model of SCI (14). The injury parameters of the device used to produce the contusion injury are shown in Table 1. No

significant differences in velocity, compression, height and time were found between agmatine-treated and vehicle-treated groups, indicating similar injuries to all animals. Open-field locomotor function was evaluated twice a week (for 6 weeks) by two blinded observers using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale, a multiple function test of locomotor outcome which provides an efficient and unambiguous locomotor rating (23-25). The method used to evaluate morphological outcome of cords following injury was similar to that described in a recent study (26, 27).

Results of the locomotor assessment are shown in Figure 1. Immediately after SCI, all animals showed bilateral hindlimb paralysis, as previously documented using the NYU impactor (23, 26). Saline-treated animals had little or no hindlimb movements 5 days post-trauma and then showed a gradual recovery over the next two weeks. By three weeks post-trauma, most saline-treated animals were stepping consistently but lacked forelimb-hindlimb coordination. There was little or no behavioral improvement in saline-treated animals between weeks 3 and 6 post-trauma. As early as 2 days post-trauma, agmatine-treated rats showed significantly more ($p < 0.001$) movement of their hindlimbs than did control animals. This difference continued throughout the duration of the survival period. Consequently, locomotor improvement observed in agmatine-treated animals was consistently greater than that observed in saline-treated animals at each time point evaluated. On day 44 after injury, the final mean BBB score for agmatine-treated animals was significantly higher than that of control animals (15.0 ± 0.83 versus 10.8 ± 0.29 , $p < 0.001$; Figure 2).

The most significant behavioral differences were observed in agmatine-treated rats from 4-6 weeks during which time they showed consistent forelimb-hindlimb coordinated movements, whereas saline-treated animals achieved only modest improvements without coordinated hindlimb movements after 4 weeks. The results have shown that agmatine significantly improves hindlimb motor function for up to six weeks following traumatic SCI. The results also showed that exogenous agmatine significantly reduces the loss of spinal cord tissue compared to saline-treated animals (Figures 3-4). Hematoxylin-, eosin- and luxol fast blue-stained sections showed that there was significant tissue loss at 44 days after injury in the saline-treated group. By contrast the total area of tissue damage in agmatine-treated animals was significantly less than that of saline-treated animals. The final mean area of tissue damage in agmatine-treated animals was thirty percent less than that found in saline-treated animals ($p < 0.001$, Figure 3). Examples of sections taken from saline- versus agmatine-treated animals are shown in Figure 3A and 3B, respectively. Note the reduced size of the cavity and sparing of white matter in the agmatine-treated section compared to the section from the saline-treated animal.

The mechanisms responsible for the neuroprotective effects of agmatine may involve the selective blockade of the NMDA receptor (14, 15) together with NOS blockade, both of which play a major role in secondary injury after brain and spinal cord injury (5, 7, 11, 28, 29). A recent study using the novel NMDA antagonist gacyclidine further underscores the importance of NMDA receptors in the pathological sequella of SCI (4). Agmatine inhibits nitric oxide synthase (16, 17), which is also an important

contributor to secondary injury after SCI (5, 12, 28, 29). Using strategies of either NMDA antagonists or NOS inhibitors has been effective in reducing the extent of tissue damage following SCI (4, 28-31). Considering the fact that agmatine has both of these properties, one would predict that agmatine might have neuroprotective effects and thus improve locomotor outcome following SCI. The results of the present study support this hypothesis.

Consistent with the results of the present study Olmos et al. (32) reported agmatine is neuroprotective against glutamate-induced neurotoxicity. Treatment with agmatine also prevents the development of opioid tolerance (33, 34), reduces infarct size after global cerebral ischemia (18), alleviates inflammatory and neuropathic pain behaviors associated with excitotoxic lesions in the spinal cord, and attenuates the extent of neuronal loss following quisqualate-induced spinal cord injury (14). Since clinically effective drug treatments for acute SCI are still needed, new agents that prevent neurological damage need to be developed. The present study suggests a therapeutic strategy for the treatment of acute spinal cord injury: single drugs with multiple targets. It is particularly noteworthy that the therapeutic targets of agmatine (NMDA receptor and NOS) have been critically linked to the pathophysiological sequella of brain and spinal cord injury. As more is learned about the temporal profile of cellular events responsible for the destruction of CNS tissue multi-action drugs (like agmatine) will need to be developed targeting early and late events in the secondary injury cascade.

In conclusion, the results of this study have shown that exogenous agmatine administered systemically for fourteen days after spinal cord injury significantly improves locomotor function. This action is consistent with a previous study showing that a single injection of agmatine following spinal nerve ligation reversed hypersensitivity indicative of pain (14). Other studies have reported that exogenous agmatine pretreatment prevents the development of opioid tolerance (33,34), reduces inflammation-induced thermal hyperalgesia (35) and reduces infarct size after global cerebral ischemia (18). Furthermore, previous studies have suggested (18) or provided evidence (14) that agmatine appears to have little detectable toxicity in rodents, suggesting a large therapeutic index. The results of the present paper extends our previous report of agmatine reduction of excitotoxic lesions and behavioral sequelae by demonstrating neuroprotection in a clinically relevant model of spinal cord injury as well as clear improvement in motor function. Importantly, improvement in locomotor function continues for at least 20 days after cessation of agmatine treatment. Collectively, this long-term efficacy and low toxicity suggests that agmatine may be a promising therapeutic candidate for treatment of acute spinal cord injury. Finally, the cumulative evidence also suggests that further investigation of agmatine with respect to therapeutic window and comparison with other currently used pharmacologic agents, e.g. MP, is warranted.

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21. **Contusion spinal cord injury:** Traumatic spinal injury was produced following a T-10 laminectomy. Animals were positioned in the weight-drop apparatus (NYU IMPACTOR). Two spinal clamps were attached to T8/T9 and T11/T12 spinal processes, respectively. A transducer was placed at the site of the muscle near the spinal column, and the impactor rod (10g) was centered above spinal segment T-10. The rod was slowly lowered until it contacted the dura, which was determined by completion of a circuit that resulted in an audible tone. The cord was then contused with the weight-drop device that released a 10g rod from a height of 12.5mm onto the exposed cord. Impact analysis, including degree of cord compression, velocity, and height of weight drop, were recorded by a preset NYU impactor software package. After trauma, rats were randomized to agmatine-treated and saline-treated groups.
22. **Drug preparation and administration:** Agmatine was purchased from Sigma (St. Louis, MO), dissolved in 0.9% saline and administered (100 mg/kg/day, i.p.) 30 min post-trauma and once daily for 14 days. The total volume injected was 1cc (saline alone or saline + agmatine).
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25. **Behavioral assessment:** Open-field locomotor function was evaluated using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale. Briefly, non-injured rats were exposed daily for one week to the behavioral testing environment in order to

acclimate them to open field exploration. Two examiners participated in the BBB evaluation and were positioned across from each other to observe both sides of the rat. During the evaluation animals were evaluated for limb, foot and toe position during locomotory movements as well as coordination between fore- and hind-limbs. Each rat was tested for 4 minutes. Postoperative open field testing for all animals occurred twice a week from Day 2 post-injury to Day 44 post-injury. Examiners were blinded to the type of treatment received by each animal.

26. C.G. Yu et al., J. Neurosug. in press.

27. **Histopathological assessment:** Forty-four days post-SCI, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with isotonic saline for 5 min, followed by a mixture of 4% formaldehyde, glacial acetic acid and methanol (FAM) 1:1:8 by volume for 30 minutes. Following perfusion, the vertebral column with the cord was immersed in FAM at 4°C for 24h. Spinal cords were then removed and embedded in paraffin in 16-18mm long blocks that contained the contusion epicenter as well as non-injured tissue at the end of each block. Each block was serially sectioned (longitudinally) at 10µm. Sections were stained with hematoxylin and eosin and luxol fast blue for histopathological analysis. Sixteen sections from the central core of the cord were studied with light microscopy, and reconstructions of the longitudinal area of tissue damage in these sections were made with the aid of an overhead projector. The area of tissue damage in each section was quantitatively determined using computer-aided image analysis (Meta Morph

Imaging System, Universal Imaging Corporation). The sum of areas in the 16 sections analyzed was used as the total area of tissue damage.

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36. **Statistical analysis:** All data are expressed as a mean \pm S.E.M. and analyzed using a commercially available computer program (StatView). For each rat, BBB scores from the two examiners were averaged together to yield one score per test session. BBB scores and total area of damage were compared between the agmatine- and saline-treated groups at each time point using one-way analysis of variance (ANOVA) and Fisher's Protected Least Significant Difference (PLSD) test. Differences were considered statistically significant at $p < 0.05$.
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FIGURE LEGENDS

FIGURE 1: Time course of locomotor recovery as measured by Basso-Beattie-Breshnahan (BBB) scores following agmatine or saline treatment. Open-field locomotor function was evaluated using the BBB locomotor rating scale. Non-injured rats were exposed daily for one week to the behavioral testing environment in order to acclimate them to open field exploration. The mean BBB scores of animals receiving agmatine (100mg/kg/day, i.p.) 30 min after trauma for 14 days are represented by the filled circles and saline-treated animals are represented by triangles. The total volume injected was 1cc (saline alone or saline + agmatine). The solid bar indicates the time period of daily agmatine administration. Data are represented as mean \pm SEM. *** p <0.001.

FIGURE 2: Mean final BBB score following agmatine or vehicle treatment. Agmatine-treated animals received agmatine (100mg/kg/day, i.p.) 30 min after trauma for 14 days. Vehicle-treated rats received saline 30 min after trauma for 14 days. Data are represented as mean \pm SEM. There was a significant difference between the final BBB scores following agmatine versus vehicle treatment. *** p <0.001.

FIGURE 3: Photomicrographs of histological sections following traumatic spinal cord injury. Injury was produced following a T-10 laminectomy. The cord was contused with the weight-drop device that released a 10g rod from a height of 12.5mm onto the exposed cord. Impact analysis, including degree of cord compression, velocity, time, and height of weight drop, were recorded by a preset NYU impactor software package. Systemic

treatment with agmatine (100mg/kg/day) starting 30 min after injury for 14 days reduced the lesion area at six weeks (A), compared with saline-treated animals (B). Note reduced area of tissue damage (A) following agmatine treatment. The area of injury was easily identified by the presence of reactive astrocytes, microglial, infiltration of inflammatory cell types (neutrophils, leucocytes) and by the presence of macrophages. Scale bar in (B) equals 400 μ m.

FIGURE 4: Area of damage following agmatine or vehicle treatment. Forty-four days post-SCI, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with isotonic saline for 5 min, followed by a mixture of 4% formaldehyde, glacial acetic acid and methanol (FAM) 1:1:8 by volume for 30 minutes. Each block was serially sectioned (longitudinally) at 10 μ m. Sections were stained with hematoxylin and eosin and luxol fast blue for histopathological analysis. Sixteen sections from the central core of the cord were studied with light microscopy, and reconstructions of the longitudinal area of tissue damage in these sections were made with the aid of an overhead projector. The area of tissue damage in each section was quantitatively determined using computer aided image analysis. Agmatine-treated animals received systemic treatment with agmatine (100mg/kg/day, i.p.) beginning 30 min after trauma and continuing daily for 14 days. The vehicle-treated animals received saline on the same schedule. Data are represented as mean \pm SEM. Compared with control, **p<0.01.

Table 1. Contusion Parameters (n=23)

	Control	Ag-treated	P ^a
Compression	1.71±0.07	1.85±0.03	NS
Height (mm)	12.48±0.10	12.62±0.08	NS
V (m/s)	0.490±0.002	0.489±0.002	NS
T (msec)	49.7±1.54	50.8±1.29	NS

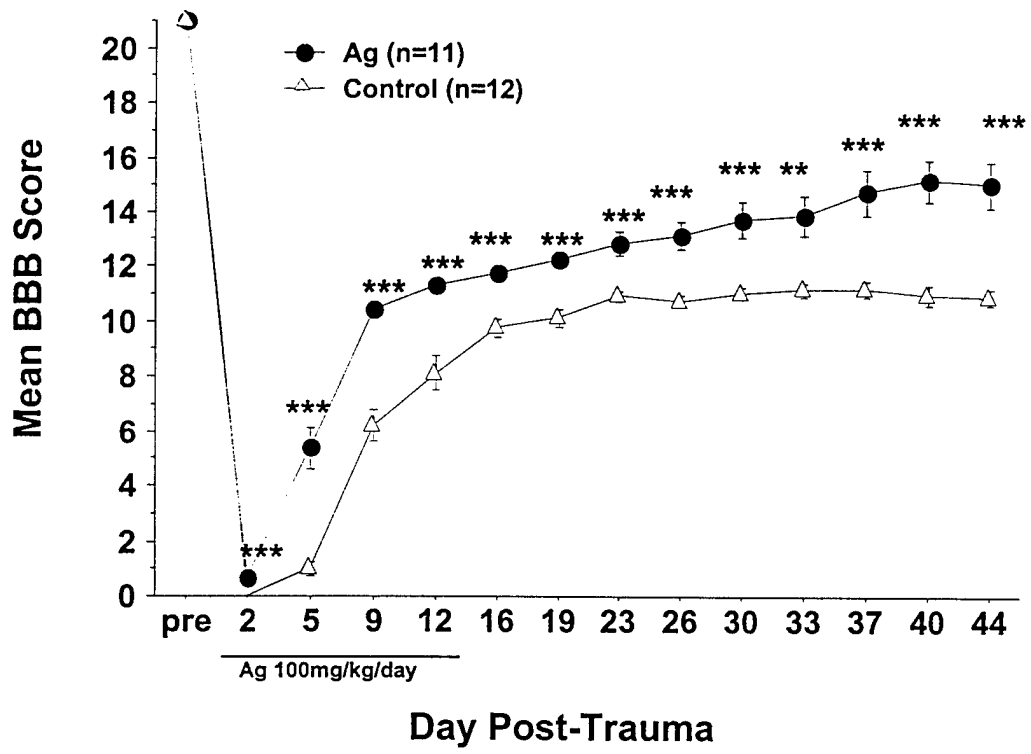
Values are means ± S.E.M.

^aANOVA and Fisher's PLSD test

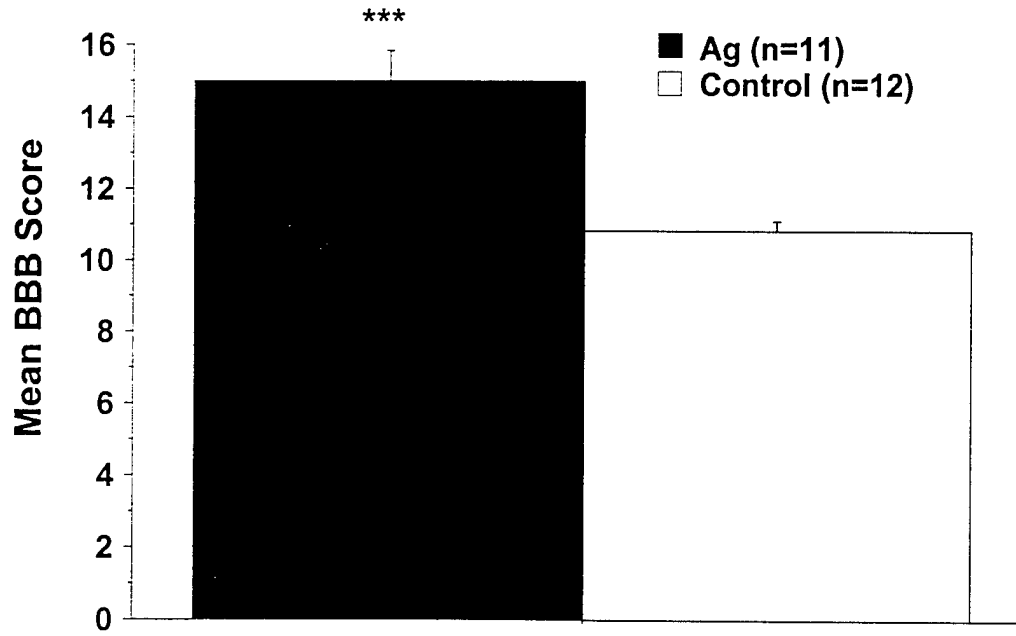
NS: Non-significant

V: Velocity

T: Time



BBB SCORE AT DAY 44

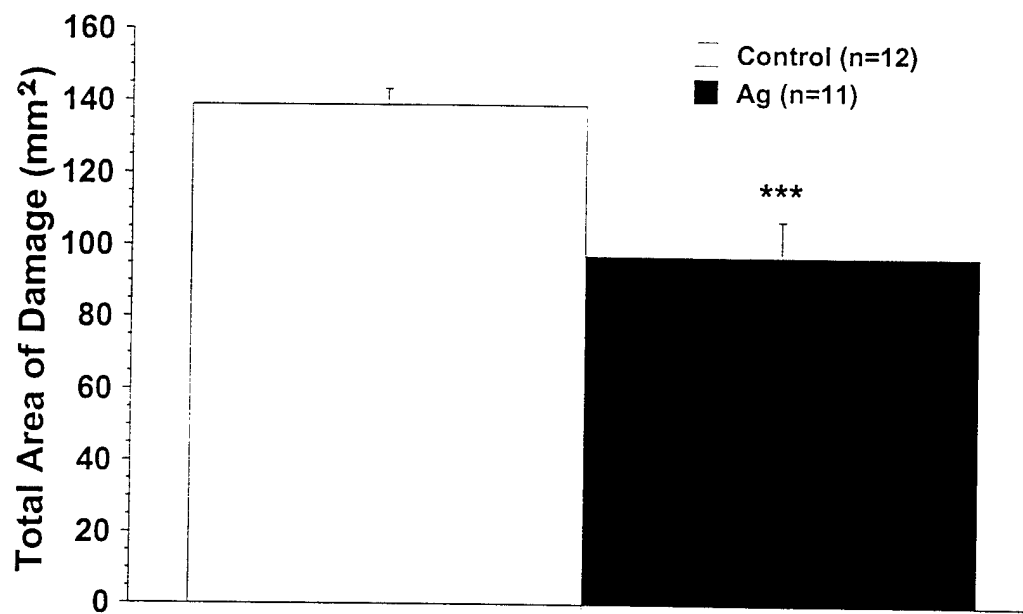


A



B





**Beneficial Effects of Modest Systemic Hypothermia on
Locomotor Outcome and Histopathological Damage
Following Contusion Spinal Cord Injury In Rats**

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Running Head: Hypothermia Following SCI

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ABSTRACT

Object: Local spinal cord cooling (LSCC) has beneficial effects following ischemic or traumatic spinal cord injury (SCI). However, the clinical application of LSCC has many technical difficulties which require special cooling devices, emergency surgery, and complicated post-operative management. The objectives of the present study were to evaluate the: (a) relationship between systemic and epidural temperature after SCI; (b) effects of modest systemic hypothermia on histopathological damage at 7 and 44 days post-SCI; and (c) effects of modest systemic hypothermia on locomotor outcome at 44 days post-SCI.

Methods: Spinal cord contusion (NYU Impactor, 12.5mm, T-10) was produced in adult rats randomly divided two groups. Group 1 (n=7, Experiment 1; n=12, Experiment 2) received hypothermic treatment (epidural temperature: 32.0-33.0°C) 30 min post-injury for 4 hours; Group 2 (n=9, Experiment 1; n=8, Experiment 2) received normothermic treatment (epidural temperature: 37.0°C) 30 min post-injury for 4 hours. Blood pressure, blood gases, and temperatures (epidural and rectal) were monitored throughout the 4 hour treatment period. Twice weekly assessment of locomotor function was performed over a 6-week survival period using the BBB locomotor scale. Seven (Experiment 1) and 44 (Experiment 2) days after injury, animals were perfused and cords were serially sectioned. The area of tissue damage was quantitatively analyzed from 16 longitudinal sections selected from the central core of the spinal cord.

Conclusions: The results showed that: (1) modest changes in the epidural temperature of the cord can be produced via systemic hypothermia; (2) modest systemic hypothermia (32-33°C) significantly protects against locomotor deficits following traumatic SCI; and (3) modest systemic hypothermia (32-33°C) reduces the area of tissue damage at both 7 and 44 days post-injury. The results lend support to the potential use of modest systemic hypothermia as a complementary treatment for acute spinal cord injury.

Key Words: Spinal Cord, Trauma, Locomotion, Hypothermia, Neuroprotection

INTRODUCTION

Since local spinal cord cooling (LSCC) was first successfully applied in experimental spinal cord injury (SCI) by Albin and colleagues¹ the protective effects of local hypothermia in many experimental models of SCI have been demonstrated.^{23, 33,9, 52, 24} However, clinical studies of LSCC are difficult to evaluate since they include only a few patients and lack randomized control groups.^{40, 54} In most experimental and clinical studies, the significant lowering (10°C) of spinal cord temperature presents many technical difficulties.

If local cooling of the spinal cord is to be used as a treatment for acute SCI, it will require special cooling devices, emergency surgery, and complicated postoperative management.^{54, 32} Modest systemic hypothermia (30-33°C) has been shown to be an effective alternative to LSCC having beneficial effects on functional and morphological outcome measures following ischemic and traumatic brain injury.^{44, 18, 21, 22, 34, 15} The conclusion from these studies is that profound lowering of CNS temperature is not necessary for neuroprotection of tissue vulnerable to damage by the effects of traumatic or ischemic injury. Systemic hypothermia, even moderate, prevents energy failure,⁷ reduces histopathological damage,^{21, 43} and diminishes free radical activity and extracellular levels of glutamate following brain injury.²⁷ Systemic hypothermia also lessens neurological deterioration resulting from brain trauma,¹² improves neurological outcome following ischemia of the rat spinal cord,^{48, 39} and reduces polymorphonuclear leukocyte accumulation following traumatic SCI.¹⁶

Clinically, systemic hypothermia has been used to prevent ischemic injuries during open heart surgery¹¹ and as a treatment modality following ischemic and traumatic injury to the brain.^{20, 26}

Moreover, mild to modest hypothermia has been demonstrated to be beneficial in preventing tissue damage following traumatic SCI.⁴⁰ The importance of such findings is that, compared to LSCC, modest systemic hypothermia provides a much simpler approach by which the cord can be cooled and thus obviates the need for acute surgical intervention. If effective, modest systemic hypothermia could provide an immediate, on-site, non-invasive therapeutic approach for acute SCI. The purpose of the present study was to investigate under controlled physiological conditions the influence of modest systemic hypothermia on locomotor outcome and histopathological damage following contusion spinal cord injury. A preliminary description of this study has been reported.⁵⁶

MATERIALS AND METHODS

Experimental procedures were approved and carried out in accordance with the Guidelines of the Animal Care and Use Committee of the University of Miami.

Experiment Design

The experimental design included an evaluation of the acute and chronic effects of systemic hypothermia on morphological and behavioral outcome measures following traumatic SCI. This was done in two separate series of experiments:

Experiment 1: (a) evaluate the relationship between systemic and epidural temperature after SCI; and (b) assess the effects of modest systemic hypothermia on histopathological damage at 7 days post-SCI.

Experiment 2: evaluate the effects of modest systemic hypothermia on locomotor outcome and histopathological damage at 44 days post-SCI.

Surgical Preparation

Under halothane anesthesia (4% in 70% N₂O, balanced with O₂), 36 female Sprague-Dawley rats (16 in Experiment 1 and 20 in Experiment 2) weighing 225-275g had their ventral neck and back shaved and scrubbed with Betadine. Using aseptic techniques, a midline ventral cervical incision was made, exposing the trachea. Under direct visual exposure, rats were intubated using a 14 gauge French endotracheal tube. The wound was closed using surgical clips. Anesthesia was then continued with halothane (2% for insertion of vascular catheters, 0.5% for trauma, hypothermia/normothermia and maintenance) via artificial ventilation at 60 cycles/min and a

tidal volume of 3ml (depending on blood gas measurements). The antibiotic Crystiben (0.01ml/100gms) was administered intramuscularly to prevent iatrogenic infection. A catheter (PE-10) was surgically placed in the tail artery for withdrawal of blood, administration of saline, and continuous monitoring of mean arterial blood pressure (MABP). An important aspect of the present study was the effort made to ensure constant physiological conditions for all animals. Physiological parameters including pO₂, pCO₂ and pH were maintained by adjusting anesthesia level, respiratory rate and tidal volume, and measured every two hours in Experiment 1 and every hour in Experiment 2 with a blood gas analyzer (Radiometer, ABL 330, Copenhagen, Denmark).

Contusion Spinal Cord Injury

Traumatic spinal injury was produced in Experiments 1 and 2 following a T-10 laminectomy. Animals were positioned in the weight-drop apparatus (NYU IMPACTOR). Two spinal clamps were attached to T8/T9 and T11/T12 spinal processes, respectively. A transducer was placed at the site of the muscle near the spinal column, and the impactor rod (10g) centered above spinal segment T-10. The rod was slowly lowered until it contacted the dura, which was determined by completion of a circuit that resulted in an audible tone. The cord was then contused with the NYU weight-drop device that released a 10g rod from a height of 12.5mm onto the exposed cord. Impact analysis, including degree of cord compression, velocity, time, and height of weight drop, were recorded by a preset NYU impactor software package.²⁹ After trauma, rats were randomized to hypothermia and normothermia treatment groups.

Hypothermia and Normothermia Treatment

Systemic temperature was controlled with a circulator water pump connected to a Plexiglas chamber with two thermal blankets. Animals were placed in the chamber and a flexible thermistor was inserted in the rectum to monitor systemic temperature. Pre-SCI, all animals were maintained at a systemic temperature of 37°C (rectal) using a feedback controlled heating blanket. Post-SCI, a small temperature probe was placed at the site of laminectomy in the epidural space to monitor epidural temperature. Beginning 30 min post-trauma, rats were placed in the Plexiglas environmental chamber in order to maintain systemic and epidural temperatures. For the normothermic group (n=9, Experiment 1; n=8, Experiment 2), the temperature was adjusted to maintain a temperature of $37.3 \pm 0.1^{\circ}\text{C}$ in Experiment 1 and $37.0 \pm 0.1^{\circ}\text{C}$ in Experiment 2 for 4 hours post-SCI. For the hypothermic group (n=7, Experiment 1; n=12, Experiment 2), the temperature was adjusted to maintain an epidural temperature of $33.1 \pm 0.2^{\circ}\text{C}$ in Experiment 1 and $32.0 \pm 0.1^{\circ}\text{C}$ in Experiment 2 for 4 hours. After each post-SCI treatment session, animals were housed in cages containing soft bedding. Animals were placed on a thermal blanket (37.0°C) until thermoregulation was reestablished and treated with Crystiben i.m. every other day for 7 days. Water bottles with extended tubes were placed to allow access to water. Food was placed inside the cage until rats were capable of reaching the standard placement in the cage top. Animals were checked daily and bladders were palpated at least twice daily and emptied as required until they regained reflex voiding.

Behavioral Assessment (Experiment 2)

Open-field locomotor function was evaluated using the BBB locomotor rating scale.³ Briefly, non-injured rats were exposed daily for one week to the behavioral testing facility in order to

acclimate them to open field exploration. Two examiners participated in the BBB evaluation and were positioned across from each other to observe both sides of the rat. Each rat was tested for 4 minutes. Postoperative open field testing for all animals occurred twice a week from Day 2 post-injury to Day 44 post-injury. Examiners were blinded to the type of treatment received by each animal.

Histopathological Assessment

At 7 days (Experiment 1) and 44 days (Experiment 2) post-SCI, rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with isotonic saline for 5 min, followed by a mixture of 4% formaldehyde, glacial acetic acid and methanol (FAM) 1:1:8 by volume for 30 minutes. Following perfusion, the vertebral column with the cord were immersed in FAM at 4^oC for 24h. The spinal cords were then removed and embedded in paraffin in 16-18 mm long blocks that contained the contusion epicenter as well as non-injured tissue at the end of each block. Each block was serially sectioned (longitudinally) at 10 μ m. Sections were stained with hematoxylin and eosin for histopathological analysis. Sixteen sections from the central core of the cord were studied with light microscopy, and reconstructions of the longitudinal area of tissue damage were made with the aid of an overhead projector and camera lucida (using 1X and 4 X objectives). The area of tissue damage in each section was quantitatively determined using computer aided image analysis. The total area of tissue damage in the 16 sections analyzed was then computed. This method of selecting sections from the middle of the cord (Fig. 1) was used because: (1) the middle sections of the cord were of high histologic quality in contrast to the top and bottom sections of the cord that were usually poor quality due to the contusive injury and/or artifacts due to histological processing; (2) sampling the same number of sections (16) from the

center of the cord ensured consistency in the way the analysis was carried out; and (3) the 16 sections analyzed contained varying proportions of white and gray matter thereby providing a representative sampling of these two anatomical regions of the cord. This method was used in preliminary studies and was found to be effective in quantifying the amount of tissue damage resulting from weight drop injury in the rat spinal cord. Importantly, this method was sensitive to differences in drug treatment protocols, and for these reasons the “central core” method of lesion analysis was concluded to be appropriate for use in the present study.

Statistical Analysis

All data are expressed as a mean \pm S.E.M. and analyzed using a commercially available computer program (StatView). For each rat, BBB scores from the two examiners were averaged together to yield one score per test session. Physiological data, BBB scores and total area of damage were compared between the normothermia and hypothermia groups at the same time point using one-way analysis of variance (ANOVA) and Fisher’s Protected Least Significant Difference (PLSD) test. Differences were considered to be statistically significant at $p < 0.05$.

RESULTS

EXPERIMENT 1

Physiological Data and Contusion Parameters

An important objective in this study was to maintain MABP, blood gases and pH within normal physiological ranges throughout all experimental procedures. As shown in Table 1, there were no significant differences in these parameters for any of the time points evaluated. There were also no significant differences observed among any of the pre- and post-contusion parameters recorded prior to and during the weight drop procedure (Table 2).

Relationships between Epidural and Rectal Temperatures

The baseline pre-weight drop rectal and epidural temperatures were $36.7 \pm 0.36^\circ\text{C}$ and $34.5 \pm 0.5^\circ\text{C}$ respectively. On average the epidural temperature pre-SCI was 2.2°C less than rectal temperature. Post-SCI rectal and epidural temperatures did not differ significantly within each treatment group, but significant differences were produced between the hypothermic and normothermic groups. In the normothermic group, rectal and epidural temperatures were maintained at $37.0 \pm 0.1^\circ\text{C}$ and $37.3 \pm 0.1^\circ\text{C}$, respectively, for a 4-hour period post-trauma. On average, the epidural temperature post-SCI was 0.3°C higher than the rectal temperature. The rectal and epidural temperatures in rats receiving hypothermic treatment were lowered to $32.2 \pm 0.1^\circ\text{C}$ and $33.1 \pm 0.2^\circ\text{C}$, respectively, 30 min post-trauma for 4 hours. On average, the epidural temperature post-SCI for this group was 0.9°C higher than the rectal temperature. The average difference between the two treatment groups was 4.8°C for rectal and 4.2°C for epidural temperatures.

Histopathological Assessment

A summary of the histopathological outcome in Experiment 1 is shown in Figures 2-3. The results demonstrated that there is significant tissue damage at 7 days after SCI in the normothermic group. Animals undergoing hypothermic treatment had significantly less tissue damage compared to the normothermic group ($p < 0.01$, Fig. 2). The effects of hypothermia resulted in a 31.7% decrease in the total area of tissue damage compared to the normothermic group. An example of the histopathological difference between the normothermic and hypothermic treatment groups is shown in the sections shown in Figure 3A and 3B. Note in the hypothermic section (Fig. 3B) there is more sparing of both white and gray matter regions of the cord.

EXPERIMENT 2

Physiological Data and Contusion Parameters

The physiological data and contusion parameters in Experiment 2 are shown in Tables 3-4. All physiological data including MABP, $p\text{CO}_2$, $p\text{O}_2$, and pH were kept within normal ranges throughout all experimental procedures. It should be noted that the MABP in the hypothermic group is higher than in the normothermic group (99.0 ± 3.7 mmHg vs 78.8 ± 3.1 mmHg, $p < 0.01$). No significant differences in contusion parameters were found between the normothermic and hypothermic groups (Table 4).

Behavioral Assessment

The results of the behavioral outcome assessment are shown in Figures 4-5. Immediately after 12.5mm SCI, animals demonstrated flaccid paralysis. In the normothermic group animals had little or no hindlimb movements 5 days post-trauma and then demonstrated a gradual recovery over the next two weeks. By 3 weeks post-trauma, most animals were stepping consistently but lacked forelimb-hindlimb coordination. There was no behavioral improvement in normothermic animals between weeks 3-6 post-trauma. Nine days after injury, ratings of locomotor performance in animals receiving hypothermic treatment were significantly increased over those exposed to normothermic treatment ($p=0.0099$). This trend continued throughout the duration of the evaluation period (Fig. 4). On day 44 after injury, the final mean BBB score for hypothermia treated animals was significantly higher than that of normothermic animals (13.3 ± 0.47 versus 10.8 ± 0.44 , $p=0.0024$; Fig. 5).

Histopathological Assessment

The results of the histopathological outcome assessment are shown in Figures 3 and 6. The results showed that there is significant tissue loss at 44 days after SCI in the normothermic group (Fig. 3C). Following hypothermia there was still a cavitory lesion within the cord (Fig. 3D), but the total area of damage was significantly less than that in rats with normothermic treatment at 44 days post-injury ($p<0.01$, Fig. 6). There was a 15.8% difference in the area of tissue damage between the hypothermic and normothermic animals. Examples of sections taken from animals in the normothermic versus hypothermic groups are shown in Figure 3C and 3D respectively. Note the reduced size of the cavity and partial sparing of white matter in the hypothermic section compared to the normothermic section.

DISCUSSION

The results of this study have shown that the NYU weight drop device which monitors compression, height, velocity and time,^{29, 4} produces reliable contusion injuries with reproducible anatomical characteristics and behavioral deficits. Our results support the conclusions from previous studies that this model is useful in testing the pre-clinical efficacy of therapeutic strategies for clinical application. In the present study, we have demonstrated the beneficial effects of modest systemic hypothermia delivered for a period of four hours after injury on functional and morphological outcome measures in the rat.

Epidural Temperature and Systemic Hypothermia

In this study temperature recordings showed that epidural temperature may be effectively lowered (32-33°C) by whole body cooling. Several approaches have been used to lower spinal cord temperature, including profound whole body hypothermia, selective cooling of the epidural or intrathecal space, or vascular perfusion of the cord.³⁹ Profound whole body hypothermia has several potential complications including the induction of undesirable changes in clotting, pulmonary, or cardiovascular function.¹⁹ On the other hand, LSCC has been shown to be effective in the lowering of spinal temperature and in the treatment of experimental SCI.^{23, 33, 9, 52, 24} Similar beneficial results have been reported in some clinical studies, but LSCC studies are difficult to evaluate since they include relatively few patients, and lack randomized control groups.^{40, 54} In most experimental and clinical studies related to hypothermia spinal cord temperature was significantly lowered (around 10°C), but the clinical application of LSCC has many technical difficulties which require special cooling devices, emergency surgery, and

complicated post-operative management.^{54, 32} Additionally, the high mortality reported in some studies remains a major concern for clinical application.

Previous findings related to the neuroprotective effects of systemic hypothermia (30-33°C) following brain ischemia and trauma may be applicable to SCI.²¹ An important question, however, is whether modest temperature changes in the spinal cord can be produced via systemic hypothermia? It is important, therefore, to determine the relationship between systemic temperature (rectal temperature) and epidural temperature for clinical practice if systemic hypothermia is to be used as an initial therapeutic strategy following spinal injury. In the present study, the epidural temperature for the hypothermic group was approximately one degree (0.9°C) higher after SCI, when compared to the systemic temperature (rectal temperature) for the same group. For the normothermic group, however, this difference was only 0.3 °C higher than the systemic temperature. These data suggest that systemic hypothermia is an effective and simple strategy for lowering cord temperature. Given the likelihood of a close relationship between epidural and systemic temperature in the clinical setting, it will be possible to use the latter as a non-invasive measure of cord temperature during the use of this technique. In the present study it was observed that the epidural temperature was significantly lower than the rectal temperature before trauma. This could be due to the exposure of the spinal cord to the environmental temperature. This finding is in accordance with a previous study.⁵⁴

Histopathological Outcome of Systemic Hypothermia

The morphological results of the present study showed that modest hypothermia has neuroprotective effects on histopathological damage following contusion SCI. The

morphological changes associated with traumatic SCI in the rat have been well characterized.^{6, 13, 46, 45} Our results have shown that following normothermic treatment, there was significant tissue damage which included hemorrhagic necrosis, cell loss, axonal swelling and vacuolization, particularly in the gray matter, at both 1 week and 6 weeks post-SCI. The acute damage at the injury epicenter was located centrally in the cord but progressed outwardly and rostrocaudally to involve adjacent areas of gray and white matter. These findings are consistent with other studies.^{6, 46} In the hypothermic group, sparing of gray and white matter occurred at both 7 days and 44 days post-SCI, and the rostrocaudal extent of total tissue damage was significantly reduced. Although no efforts were made to distinguish between the protective effects of hypothermia on white versus gray matter, the effects observed on locomotor function, i.e. BBB scores, suggests hypothermic treatment had a significant effect on white matter regions of the cord.

In order to establish a meaningful relationship between hypothermic and normothermic groups as well as determine if there exists a morphological correlate for the behavioral improvement observed with hypothermia, it was imperative to quantify the amount of tissue damage for animals undergoing different post-SCI treatments. Several investigators have developed methods to quantify the amount of tissue damage following SCI including: lesion volume;^{13, 14} and (2) percentage of spared spinal cord tissue.^{41, 6, 37} In the present study we used a histopathological method that relied upon the selection of 16 longitudinal sections from the middle of the cord. The major advantages of this approach include: (1) the selected sections from the central core of the cord included representative regions of white and gray matter; (2) the use of longitudinal sections made it possible to visualize the full rostrocaudal extent of the injury; (3) the central

core region is typically the region of the cord most affected by contusion injury; and (4) the middle sections of the cord were of high histologic quality in contrast to top and bottom sections of the cord that were usually poor quality because of the contusion injury and/or artifacts due to histological processing. Using the “core” method of analysis our results support the conclusion that modest systemic hypothermia has beneficial effects on histopathological damage following contusion SCI. In the present study, the mean sum of damaged areas from hypothermic animals were significantly less than from animals in the normothermic group at both 1 week and 6 weeks post-SCI. Further validation of this technique comes from recent studies in our lab which have shown the beneficial effects of agmatine, a NMDA antagonist and iNOS inhibitor, on morphological outcome following SCI,⁵⁶ and the detrimental effects of hyperthermia on a similar morphological outcome measure (Yeziarski et al., unpublished observations).

Behavioral Recovery and Systemic Hypothermia

The behavioral results of the present study demonstrated that the time course of locomotor recovery in the normothermic group was similar to previous reports in the rat.⁴ A number of methods have been devised for the assessment of residual neurological function following experimental SCI. The most widely used are modifications of Tarlov’s score, which includes an assessment of spontaneous locomotion,⁵¹ and the inclined plane score described by Rivlin and Tator.⁴⁷ Several other tests have been utilized, such as grid walking,¹³ footprint analysis,³⁶ the response to paw pinch and reflex righting.²⁵ The BBB locomotor rating score is a multiple function test of locomotor outcome which provides an efficient, expanded and unambiguous locomotor rating.^{3, 4} BBB scores differ from other locomotor scoring systems in several respects. First, the score is not a summation of component behaviors. Each BBB score requires fulfillment

of a unique set of criteria. Second, the scores encompass many behavioral traits and represent a detailed characterization of rat locomotor function. Application of the BBB behavioral test allowed us to effectively evaluate the neuroprotective effects of hypothermia against motor dysfunction following contusion SCI. In this study, one week after injury, ratings of locomotor performance in rats receiving hypothermia treatment were significantly increased over those exposed to normothermic treatment. This trend continued throughout the duration of the study and the difference became larger over 6 weeks. On day 44 after injury, the final mean BBB score for animals in the hypothermic group was significantly higher than that of normothermic rats. These results are consistent with previous studies showing systemic hypothermia to be protective against brain and spinal cord ischemia caused by vascular occlusion.³⁹ To our knowledge this is the first systematic evaluation showing the beneficial effects of modest (32-33°C) hypothermia on morphological and behavioral outcomes following traumatic SCI.

Mechanisms of Systemic Hypothermia-Mediated Protection

The mechanisms of systemic hypothermia-mediated protection against histopathological damage and locomotor outcome following contusion SCI are not understood. Several hypotheses have been suggested.²² Early speculation was based on the reduction in tissue metabolic and oxygen requirements that occurs when CNS tissue is cooled.⁴⁰ Since the mid 1950s and latter 1970s hypothermia has been demonstrated to protect against the loss of phosphocreatinine and the accumulation of lactate, decrease of cerebral oxygen consumption as well as lowering the rate of cerebral ATP depletion following the interruption of cerebral circulation.^{49, 30, 31, 35, 42} During the 1980s and 1990s, experimental studies and clinical observations showed that brain and spinal cord lesions are greatly enlarged by secondary injury. Although the molecular and cellular

mechanisms underlying these events are still not clearly understood, evidence suggests that hypothermia influences several changes responsible for secondary injury. For example, hypothermia attenuates oxygen free radical production,²⁷ suppresses release of the excitatory amino acid neurotransmitter glutamate,^{39, 27} reduces intracellular calcium overload,² prevents loss of microtubule-associated protein-2⁵⁰ and the delayed induction of iNOS,¹⁶ diminishes induction of IL-1 β mRNA,²⁸ and lowers lipid peroxidation.⁵³ In a recent study we have shown that another potential contributing factor to the effects of hypothermia is the reduction of post-traumatic inflammation.¹⁷ In this study we observed a significant reduction in the accumulation of polymorphonuclear leukocytes following hypothermic treatment. In addition, significant reductions in spinal cord blood flow (SCBF) have been documented following traumatic SCI by various investigators.⁴⁰ Cooling the spinal cord could therefore have an effect on the degree of posttraumatic hypoperfusion.⁴⁰ In the present study, hypothermic rats had a higher MABP than normothermic rats. Similar results have been reported in another study.³⁸ The increased MABP may have improved SCBF in injured regions with impaired autoregulation, thereby, reducing ischemia in the early post-trauma period.³⁸

Although the mechanism for the beneficial effects of hypothermia are not entirely understood, the fact remains that this intervention has a significant impact on the pathological and functional state of injured CNS tissue. While these effects lend encouraging support for clinical application, of equal importance are the effects of hypothermia on specific components of excitotoxic and inflammatory cascades. These effects provide important insight into the identification of potential therapeutic targets for the treatment of acute spinal injury which further adds to the importance of using this experimental protocol. To further understand the full scope of effects

provided by hypothermia it will be important in the future to determine whether increasing the duration of post-injury hypothermic treatment offers any additional beneficial effects, and whether it is possible to enhance the pharmacological effects or widen the therapeutic window of other interventions such as methylprednisilone¹⁰ or IL-10.⁸ If the effects of immediate post-SCI hypothermia include an extension of the therapeutic window for these interventions this would be an important benefit for patients unable to get immediate pharmacological or surgical intervention.

Conclusions

It is concluded that: (1) modest temperature changes in the spinal cord can be produced via systemic hypothermia; (2) modest systemic hypothermia (32-33°C) significantly protects against behavioral abnormalities following traumatic SCI; (3) modest systemic hypothermia reduces the area of tissue damage at both 7 and 44 days post-injury. The results lend support for the use of modest systemic hypothermia as a possible non-invasive treatment for acute spinal cord injury.

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FIGURE LEGENDS

FIGURE 1: The location of sections sampled for the quantitative analysis of tissue damage resulting from weight drop injury. Sixteen longitudinal sections from the middle of the cord were selected for analysis. (A) Longitudinal representation of the cord showing the rostrocaudal extent of the primary (black) and secondary (dashed lines) injury as well as intact tissue at each end of each block. The area highlighted by the diagonal lines represents the location of sections sampled for analysis. (B) Cross section of cord showing the location of sections selected for analysis. Each section contained a representative sample of white and gray matter. The middle sections of the cord were of high histological quality in contrast to the top and bottom sections that were usually poor quality due to contusion injury and/or artifacts due to histological processing.

FIGURE 2: Histogram showing the area of tissue damage following hypothermic or normothermic treatment 7 days after weight drop injury. The hypothermic group (n=7) received treatment (32-33°C) 30 min after trauma for 4 hours. The normothermic group (n=9) received treatment (37°C) 30 min after trauma for 4 hours. Data are presented as mean \pm SEM **p<0.01.

FIGURE 3: Photomicrographs of histological sections following contusion injury of the spinal cord. Systemic treatment with modest hypothermia starting 30 min after injury for 4 hours significantly reduced the lesion area at 1 week (B) and 6 weeks (D), compared with control (normothermia) at 1 week (A) and 6 weeks (C), respectively. Note reduced area of tissue damage (B) and size of cavity (D) following hypothermia. Scale bar in (C) equals 280 μ m.

FIGURE 4: Time course of locomotor recovery as measured by B-B-B scores following hypothermic or normothermic treatment. The mean B-B-B scores of animals receiving modest hypothermia (32°C) 30min after trauma for 4 hours are represented by the filled circles and normothermia (37°C) are represented by triangles. Data are presented as mean \pm SEM. *p<0.05, **p<0.01.

FIGURE 5: Final B-B-B score 44 days following weight drop injury and normothermic or hypothermic treatment. The hypothermic group received treatment (32°C) 30 min after trauma for 4 hours. The normothermic group received normothermia (37°C) 30 min after for 4 hours. Data are represented as mean \pm SEM. There was a significant difference between the final B-B-B scores following hypothermic versus normothermic treatment. **p<0.01.

FIGURE 6: Area of damage following weight drop injury and hypothermic or normothermic treatment. The hypothermic group received treatment (32-33°C) 30 min after trauma for 4 hours. The normothermic group received treatment (37°C) 30 min after trauma for 4 hours. Data are represented as mean \pm SEM. There was a significant difference in the final area of damage for the two treatment groups. **p<0.01

Figure 1A

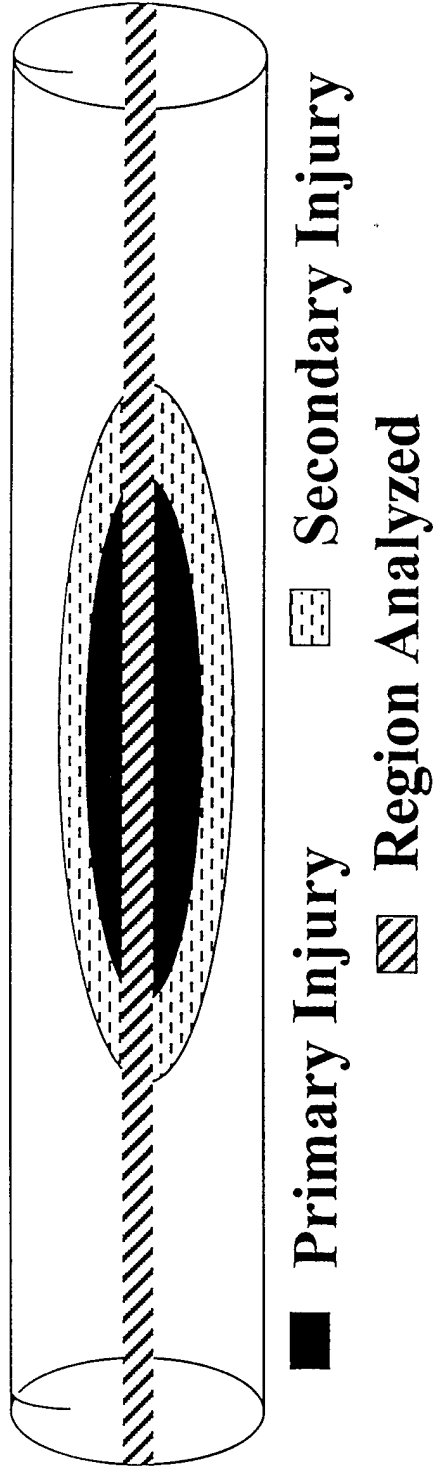
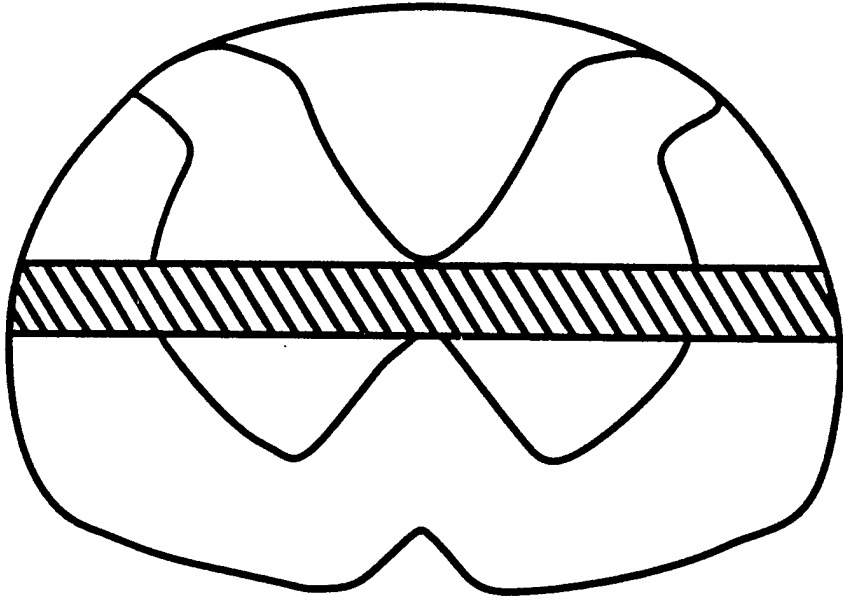


Figure 1B



AREA OF DAMAGE AT DAY 7

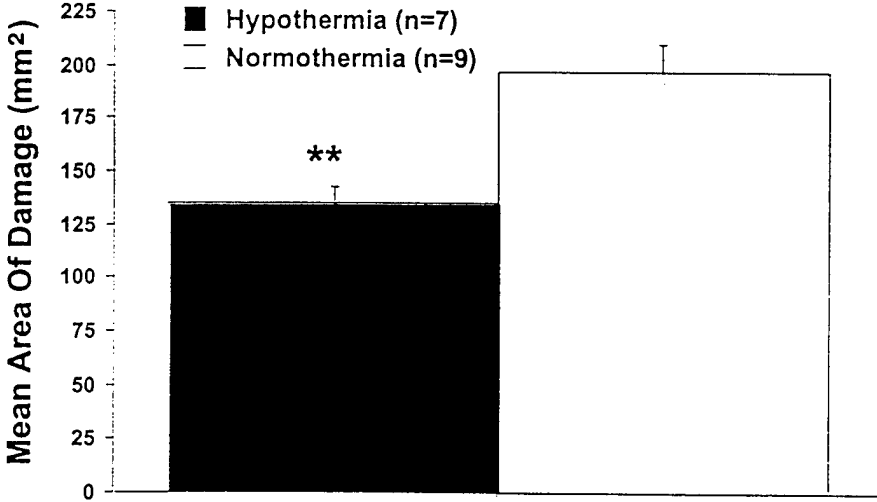
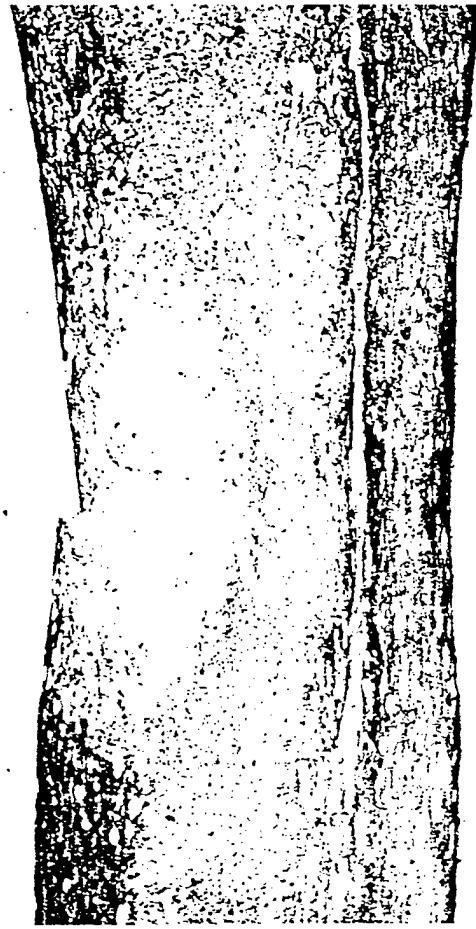
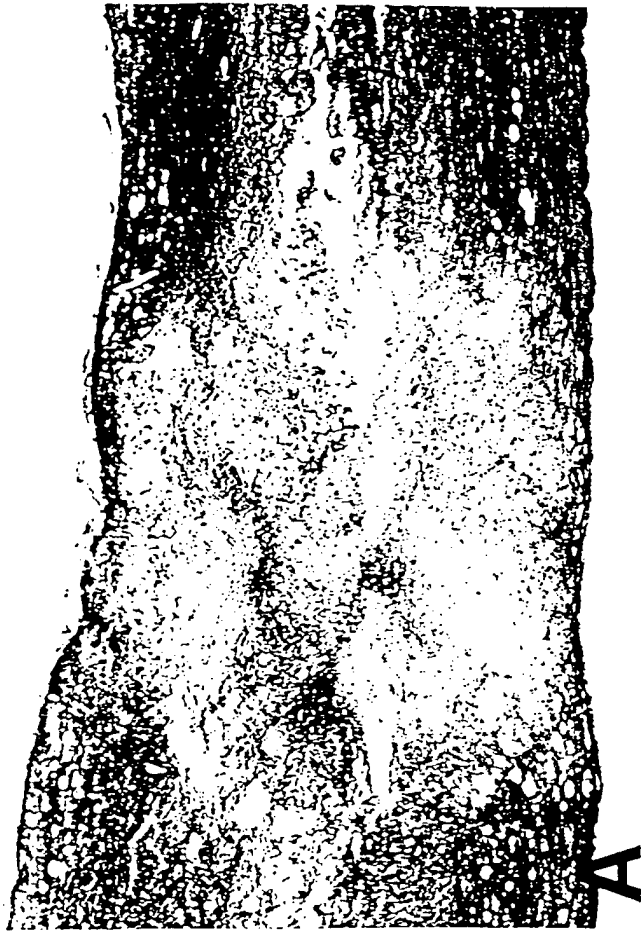


FIGURE 2

FIGURE 3



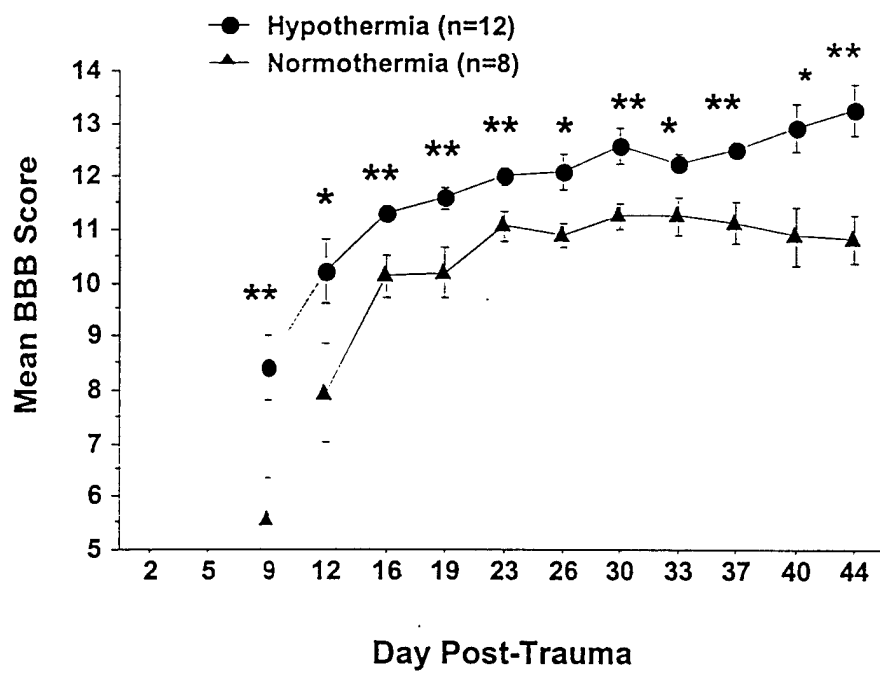


FIGURE 4

B-B-B SCORE AT DAY 44

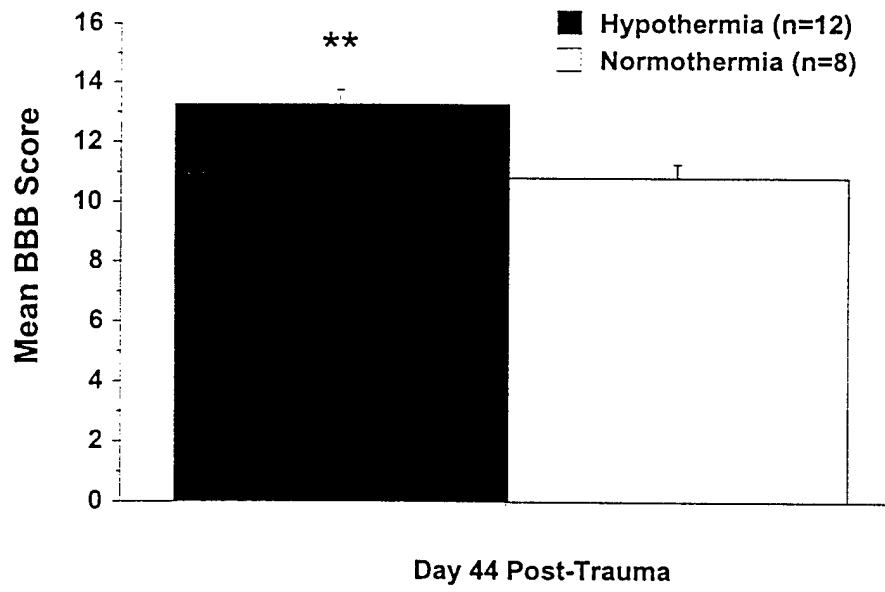


Figure 5

FIGURE 6

AREA OF DAMAGE AT DAY 44

