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## A Novel Dehydroepiandrosterone Analog Improves Functional Recovery in a Rat Traumatic Brain Injury Model

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### Abstract

The purpose of this study was to investigate the efficacy of a novel steroid, fluasterone (DHEF, a dehydroepiandrosterone (DHEA) analog), at improving functional recovery in a rat model of traumatic brain injury (TBI). The lateral cortical impact model was utilized in two studies of efficacy and therapeutic window. DHEF was given (25 mg/kg, intraperitoneally) at the initial time point and once a day for 2 more days. Study A included four groups: sham injury, vehicle treated ( $n = 22$ ); injured, vehicle treated ( $n = 30$ ); injured, pretreated (5–10 min prior to injury,  $n = 24$ ); and injured, posttreated (initial dose 30 min postinjury,  $n = 15$ ). Study B (therapeutic window) included five groups: sham injury, vehicle treated ( $n = 17$ ); injured, vehicle treated ( $n = 26$ ); and three posttreatment groups: initial dose at 30 min ( $n = 18$ ), 2 h ( $n = 23$ ), or 12 h ( $n = 16$ ) postinjury. Three criteria were used to grade functional recovery. In study A, DHEF improved beam walk performance both with pretreatment (79%) and 30-min posttreatment group (54%;  $p < 0.01$ , Dunnett vs. injured vehicle). In study B, the 12-h posttreatment group showed a 97% improvement in beam walk performance ( $p < 0.01$ , Dunnett). The 30-min and 12-h posttreatment groups showed a decreased incidence of falls from the beam, which reached statistical significance ( $p < 0.05$ , Dunnett). Tests of memory (Morris water maze) and neurological reflexes both revealed significant improvements in all DHEF treatment groups. In cultured rat mesangial cells, DHEF (and DHEA) potently inhibited interleukin-1 $\beta$ -induced cyclooxygenase-2 (COX2) mRNA and prostaglandin (PGE2) production. In contrast, DHEF treatment did not alter injury-induced COX2 mRNA levels in the cortex or hippocampus. However, DHEF (and DHEA) relaxed *ex vivo* bovine middle cerebral artery preparations by about 30%, with an IC<sub>50</sub>  $\approx$  40  $\mu$ M. This was a direct effect on the vascular smooth muscle, independent of the endothelial cell layer. Fluasterone (DHEF) treatments improved functional recovery in a rat TBI model. Possible mechanisms of action for this novel DHEA analog are discussed. These findings suggest an exciting potential use for this agent in the clinical treatment of traumatic brain injury.

### Keywords

cyclooxygenase-2; neurosteroid; rat traumatic brain injury

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## INTRODUCTION

THE PRIMARY INJURY of brain trauma sets into motion cascades of responses which may be beneficial or lead to secondary injury. The acute release of cytokines (Morganti-Kossmann et al., 1997, Soares et al., 1995; Yan et al., 1992) and vasoactive factors (Ellis et al., 1989) alters cerebrovascular reactivity (Robertson, 1996; Sioutos et al., 1995) and initiates the central inflammatory response. However, if not properly regulated, these initially beneficial responses may ultimately become harmful (Strauss, 1998). No pharmacotherapy has been proven effective for treatment of the primary and secondary insults of traumatic brain injury (TBI).

The goal of the present study is to investigate a new class of drugs, the dehydroepiandrosterone (DHEA) analogs, for the treatment of experimental TBI. Lowered endogenous DHEA levels in humans and lab animals have been associated with a variety of undesirable physiological processes including aging, cognitive decline, aggression, obesity, cardiovascular disease, immune dysfunction, and changes in basal metabolism (Majewska, 1995). Exogenous DHEA steroids were shown to reduce the severity of symptoms in adjuvant-induced arthritis (Williams, 1997) and block the interleukin (IL)-1 $\beta$ -mediated rise in prostaglandin production in rat mesangial cells (A.G. Schwartz et al., unpublished results). However, DHEA is androgenic which may limit its usage in the treatment of patients. This prompted the development (by Dr. A.G. Schwartz, Dr. J.R. Williams, both at Temple University, and Dr. M. Lewbart) of a nonandrogenic fluorinated analog, 16 $\alpha$ -fluoro-5-androsten-17-one, fluasterone or DHEF (Fig. 1). DHEF demonstrates similar biological activity to DHEA, without the undesirable side effects linked to androgenic steroids, glucocorticoids, or nonsteroidal antiinflammatory drugs (NSAIDs). DHEF did not demonstrate the androgenic, estrogenic, or peroxisome-proliferating effects of DHEA in rodents and humans, yet did retain the antineoplastic and anti-diabetic effects of DHEA (Ratko, 1991; Schwartz and Pashko, 1993; Schwartz et al., 1989). Fluasterone is currently in Phase I/II clinical trials for autoimmune (e.g., psoriasis) and metabolic (e.g., Syndrome X hypertriglyceridemia) diseases.

There is much evidence that free radicals exacerbate tissue damage and inflammation after brain injury (Hall, 1996). The DHEA class of steroids inhibits production of reactive oxygen and nitrogen intermediates, possibly by uncompetitively inhibiting glucose-6-phosphate dehydrogenase (G6PDH), thereby suppressing the formation of NADPH, a critical reductant in free radical formation (Gordon et al., 1995; Pashko et al., 1991; Raineri and Levy, 1970; Schwartz and Pashko 1993, 1994). DHEA also effectively inhibits nitric oxide (NO) production (Mei et al., 1995); presumably by limiting NADPH production, a required co-factor for nitric oxide synthase activity (Marletta, 1994). The pentose phosphate pathway, of which G6PDH is the rate-limiting step, is also a primary source of nucleic acid backbones, necessary for cellular proliferation. In models of peripheral and central inflammation, as well as in human subjects, DHEA, DHEF, and other analogs have proven beneficial in reducing cellular proliferation, attenuating free radical formation, and altering inflammatory-mediated gene expression (Aragno et al., 2000; Boccuzzi et al., 1997; Dersken, 1998; Morales et al., 1994; Van Vollenhoven, 1994, 1995, 1998). These findings are consistent with the hypothesis that DHEA steroids suppress inflammation via G6PDH inhibition. In addition, DHEA and related pregnane and androstane steroids can act as neuroactive neurosteroids (Baulieu and Robel, 1996). As allosteric inhibitors of GABA<sub>A</sub> receptor function, and weak agonists at the glutamate receptor sigma subunit (Majewska, 1995; Maurice et al., 1997) these compounds may modulate neuronal excitability. DHEAs block pharmacologically induced amnesia in mice (Flood et al., 1988a,b), and improve memory on automatized tasks in humans (Wolkowitz et al., 1995). This concept is supported by earlier findings that GABA agonists (barbiturates, benzodiazepines) produce amnesia, whereas GABA antagonists (picrotoxin, pentylenetetrazole) reverse amnesia and improve memory (Breen, 1961; Irwin, 1966). For example, chronic administration of the

benzodiazepine antagonist flumazenil can prolong the life span and improve memory in rats (Marczynski et al., 1994).

In addition, DHEAs may increase hypothalamic and possibly cortical levels of serotonin as well as potentiate NMDA-induced noradrenaline release from hippocampal slices, apparently by stimulating glutamatergic  $\sigma$  receptors (Majewska, 1995; Maurice et al., 1997). These latter mechanisms, may account for the reported antidepressant effects of DHEA in humans (Wolkowitz et al., 1995), and may additionally contribute to its potentiating effect on memory, in which noradrenaline plays a pivotal role (Majewska, 1995; Sara et al., 1994; Stanton and Sarvey, 1985). Neurosteroids may play a dynamic role in shaping neuronal architecture and, as such, may have critical functions in learning, memory and neuronal survival during or after injury to the brain. Thus, DHEF may have pleiotropic mechanisms of action in recovery from TBI. Our current work is focused on determining the efficacy of DHEF in experimental head injury, and characterizing its therapeutic window.

## MATERIALS AND METHODS

### Traumatic Brain Injury Model

The rat lateral controlled cortical impact (LCI) model of TBI was used to induce moderate levels of injury. LCI results in a temporary loss of strength and coordination in the limbs contralateral to the injury, significant retrograde amnesia, a loss of cortical and subcortical neurons, and induction of inflammatory responses. LCI produces focal cortical and subcortical damage similar to human closed head injury, with little involvement of the brainstem. The relevance of this rat model of TBI to clinical head trauma has been validated by several groups, reproducing common cognitive-behavioral outcome measures and histopathological findings (Dixon et al., 1991; Hamm et al., 1992; McIntosh et al., 1989).

**Animals**—Male Sprague-Dawley rats (Harlan), weighing 300–400 g, were studied. Animals were cared for in accordance with U.S. Public Health Service regulations. All protocols were approved by the Temple University Institutional Animal Care and Use Committee.

**Surgical preparation**—Rats were preanesthetized (~2% isoflurane), scalp shaved, and given oxygen with 0.75% isoflurane through a fixed nose cone on a stereotaxic platform. The cranium was exposed and a craniectomy made laterally (left side), midway between lambda and bregma, between the central suture and the left temporal ridge using a 6-mm trephine. The intact dura was subjected to a piston impact of 3.0 mm depth, 4 m/sec velocity, and 100 msec duration. The animal was taken off anesthesia and assessed for exclusion criteria. To be included in the study, animals needed to regain the pinna and corneal reflexes within 2–4 min, awaken within 4–5 min, and regain their righting response within 9 min after removal from anesthesia.

**Experimental treatment groups**—For these studies, animals were treated immediately before or at various times after LCI TBI (Table 1). The DHEF and the Emulphor-saline vehicle were provided by Dr. Arthur Schwartz. Sham/vehicle and injured/vehicle groups received intraperitoneal injections 10 min before injury, followed by doses at 24 and 48 h postinjury. The injured/DHEF groups received DHEF (25 mg/kg, 8.75 mg/mL) at the initial time point (10-min pretreatment, or 30-min, 2-h, or 12-h posttreatment) followed by doses at 24 and 48 h postinjury.

### Functional Evaluations

All neurological and behavioral assessments were performed before and 3 days after injury. The observer was blinded to the treatment group information. Improvements were calculated

as the difference between injured/DHEF-treated and injured/vehicle groups divided by the difference between sham and injured/vehicle groups ( $\times 100\%$ ).

**Behavioral assessments**—Behavioral assessments included an open field test and the Morris water maze. To test exploratory behavior, animals were placed in an open field (30 cm  $\times$  61 cm  $\times$  20 cm deep) and observed for 2 min. The number of rearings and transits to each corner were summed for an activity score. The water maze was used to assess declarative memory function. Pre-training and training consisted of 120-sec trials, starting at four alternating compass points in a 2-m circular tank with opaque water (white tempera paint) and a submerged platform halfway from the wall to the center of the maze. After reaching the platform, animals remained there for 60 sec to become familiar with the extra-maze cues placed on the surrounding walls. Animals were pretrained (nine trials) the day before injury, and trained (10 trials) the day of injury, finishing 1.5 h before the anesthesia. Water maze scoring used two concentric zones around the platform site (40 and 100 cm diameter); time spent in each zone was measured during a 120-sec videotaped probe trial (with the platform removed). The water maze score was calculated as the sum of the weighted times in each zone, adjusted using the inverse proportion of the zone area compared to the whole tank (multipliers: inner zone = 24, outer zone = 4). This approach weights the time in a particular concentric zone by the (inverse) probability that the animal would be found in that area of the maze by chance. Thus, the smaller the zone, the greater the weighting factor, and vice versa. This nonarbitrary weighting should yield more sensitive and accurate results when access to a computerized analysis package is limited.

**Neurological reflexes**—Neuroscores were based on three tests of limb reflexes (McIntosh et al., 1989). The tests were contraflexion (forelimb and head flexion in response to anticipation of falling), hind limb extension (in response to repetitive raising and lowering by the tail), and lateral pulsion (test of strength and coordination upon attempts to roll the animal onto its back). Scores of 0–4 (4 being best) were given for left and right limbs separately for a total of 24 points on the three tests.

**Beam walk test**—The beam walk is a balance beam task with both cognitive and motor components. Animals were trained to escape an unpleasant pair of stimuli (200-W light bulb and 90-dB white noise) by running 1 m along a 2.5-cm beam with several posts mounted on alternating sides, and entering a black box (the stimuli were turned off once the animal entered the box). The time taken to enter the box was the outcome measure (0–120 sec). In addition, in Study B, if animals were unable to complete the task or fell from the beam, these were recorded and studied separately.

**Cerebral vessel reactivity**—We optimized an *ex vivo* preparation of bovine middle cerebral arteries (bMCA) to test DHEA and DHEF vasoactivity (Wendling and Harakal, 1987, 1991; Wendling et al., 1994). With prior approval of the US. Dept. of Agriculture Meat Inspection Program, bMCA were obtained from freshly slaughtered animals. The arteries were isolated, immersed in physiologic saline solutions (PSS) at 1°C, transported to the laboratory, and cleaned of fat and debris. For isometric tension experiments, arteries were cut into rings of uniform width (2.5 mm) and mounted vertically on two L-shaped wire holders between a wire anchor and a Grass isometric transducer. The rings were maintained in 40–50 mL of oxygenated PSS ( $[\text{CaCl}_2] = 1.2 \text{ mM}$ , pH 7.4, 37°C) over a 2–3-h equilibration period. Constrictors were used to gradually increase resting tension to optimal (0.5 g) for the bovine cerebral arteries. Isometric tension was recorded simultaneously on Radnoti Model TRN005 or TRN006 instrumentation amplifiers and on a Kipp and Zonen Model BD112 recorder, linked to the amplifiers via an 8-channel multiplexer (Instech Laboratories). For endothelial stripping experiment, arteries were opened into strips approximately 1–1.5 cm long. The endothelial

layer was carefully stripped, and each tissue was weighed, mounted on two wire holders, and equilibrated at 0.5 g tension, as above. After a 3-h equilibration in PSS at 37°C, the strips were treated with vehicle, DHEA, or DHEF for 45 min. In all experiments, arterial rings or strips were selected at random to receive a treatment. Results were expressed as mean + SEM ( $n \geq 6$  rings or strips for each variable). The inter group comparisons were examined using analysis of variance, and the Student's *t*-test or the Newman-Keuls multiple range *t*-test.

**In vitro inflammation model**—Kidney-derived rat mesangial cells (kind gift of Dr. Jeffrey Kreisberg) were cultured in RPMI medium containing 20% fetal bovine serum. After reaching confluence, cells were incubated in serum-free medium for 48 h. The inflammatory cytokine IL-1 $\beta$  (1 ng/mL) was added (with or without DHEA steroids), and the cells were harvested after 4 h by scraping into 5 M guanidine thiocyanate, 0.1 M EDTA, pH 8.

**Measurement of COX2 mRNA levels**—COX2 gene expression was measured in a lysate ribonuclease protection assay (Strauss et al., 2000). The cell lysates were either from cultured rat mesangial cells (above) or from frozen rat brain micropunches sonicated in 6 M guanidine thiocyanate, 0.1 M EDTA, pH 8. Aliquots (40  $\mu$ L,  $\sim 2 \times 10^5$  cells) were analyzed using cRNA probes to COX2 and cyclophilin (Strauss et al., 2000). Comparisons were made on mRNA levels normalized for protein or cyclophilin in each sample. Results were expressed as mean  $\pm$  SEM ( $n = 4$ –6 per group).

**Statistical analyses**—Statistical evaluation of data was performed using chi-square, and analysis of variance with Dunnett and Scheffé post hoc tests. For all analyses, the injured/vehicle group was used as the positive control. Probability values less than 0.05 were considered statistically significant to reject the null hypothesis that the group means were equal.

## RESULTS

Fluasterone (DHEF, 25 mg/kg ip., 3 days) treatments initiated before or up to 12 h after TBI improved functional recovery at 3 days postinjury. The open field test was used as a negative control, to ensure that the injury did not alter the animals' exploratory behavior. No injury-induced changes in open field behavior were observed among any of the groups. This treatment induced weight loss greater than that caused by the cortical contusion injury alone (Table 1). DHEF administered at lower doses (8.3 and 2.5 mg/kg ip., initiated 0.5 h postinjury) did not improve behavioral recovery and did not produce additional weight loss (data not shown).

### Improved Beam Walk Performance

DHEF treatments, initially given before or after injury, improved beam walk performance measured 3 days postinjury. In Study A, DHEF produced 79% and 54% improvements in beam walk latency in the pretreatment and 0.5-h posttreatment groups, respectively ( $p < 0.01$ , Dunnett, Fig. 2A). In Study B, both latency and ability to complete the beam walk were examined. DHEF initially administered 0.5 h and 12 h (but not at 2 h) postinjury markedly improved performance 3 days after TBI compared to the injured, vehicle treated littermate controls (Fig. 2B). The 12-h posttreatment group was the only group that showed no significant difference from sham animals on this task. DHEF posttreatments (0.5 and 12 h) also reduced the number of falls on the beam walk task, compared to injured controls (Table 1,  $p < 0.05$ ,  $\chi^2$  test).

### Improved Neurological Reflexes Measured Using Neuroscore

Neurological reflexes improved significantly in both treatment groups of Study A (29%, Fig. 3A). All treatment groups in Study B also showed improved recovery compared to injured, vehicle-treated controls (37–63%). The group neuroscores for Study B were as follows: sham,

19.9 ± 1.8; injured, 17.3 ± 1.3; DHEF/0.5, 19.0 ± 1.2; DHEF/2, 19.1 ± 1.2; DHEF/12, 14.5 ± 0.6. The raw data revealed a trend toward intergroup differences *before* injury ( $p = 0.0623$ ), as well as a trend toward intergroup differences at 3 days postinjury ( $p = 0.0896$ ). Thus, to specifically extract the injury-induced changes, the difference between pre- and postinjury neuroscores were evaluated as the dependent variable ( $p = 0.0259$ , Fig. 3B). This was not the case in Study A; preinjury differences were not significant ( $p = 0.7283$ ) and both raw (Fig. 3A) and difference neuroscores showed statistically significant treatment effects at 3 days postinjury ( $p = 0.0001$ ). The 12-h posttreatment group seemed to improve more than the other groups, showing no significant difference from shams.

### Declarative Memory Measured in the Morris Water Maze

Water maze performance similarly revealed statistically significant improvements (45%) in the pretreatment and 0.5-h posttreatment groups of Study A ( $p < 0.05$ , Scheffé, Fig. 4A). In Study B, all treatment groups performed better than injured/vehicle (8–36%,  $p < 0.05$ , Dunnett), with the 12-h posttreatment group again improving most ( $p < 0.01$ , Dunnett, Fig. 4B). To dissociate possible motor deficits at this time, the swim speed (time to cross the maze diameter) was determined at 3 days postinjury during the testing regime. There were no differences in swim speed between sham and any of the injured groups.

### Modulation of COX2 mRNA Induction

COX2 is an early effector in the central inflammatory pathway and is induced by cytokines. To test the antiinflammatory properties of DHEF, an *in vitro* culture of rat mesangial cells was utilized, as previously described (Feng, 1995). Interleukin-1 $\beta$  (1 ng/mL) increased the level of COX2 mRNA by threefold in these cells. Treatment with DHEF (2.5  $\mu$ M) or DHEA (data not shown) completely attenuated interleukin-1 $\beta$ -mediated induction of COX2 mRNA (Fig. 5).

In rats, cerebral COX2 mRNA and protein are induced within 2 h of TBI, and continue to be elevated for at least 3–7 days postinjury (Strauss et al. 2000). Surprisingly, DHEF did not modulate the *in vivo* expression of COX2 in the injured cortex or hippocampus at any time following TBI (Fig. 6).

### Modulation of Cerebral Vasoreactivity

To investigate possible mechanisms of action, we tested whether DHEF or DHEA affected cerebral blood vessel constriction or dilation. Serial addition of DHEF or DHEA to K<sup>1</sup> constricted bovine middle cerebral artery preparation (bMCA) caused direct vasodilatation with IC<sub>50</sub>  $\approx$  40  $\mu$ M (Fig. 7). The site of action of this vasoactivity was further investigated by removal of the endothelial cell layer from the bMCA preparations. At their IC<sub>50</sub>, the relaxation produced by DHEF and DHEA was preserved, indicating a direct effect on the vascular smooth muscle cells (data not shown).

## DISCUSSION

The DHEA analog fluasterone (DHEF) improved neurological and cognitive recovery in a rat model of lateral cortical contusion traumatic brain injury. No changes were observed in the open field test, a control to confirm that the injury was limited to the somatosensory cortex and underlying structures. Changes were most pronounced on the beam walk test which is a complex coordination task with cognitive components. Not only did the time latency improve in the treatment groups but the animals also revealed a decreased incidence of falls from the beam compared to their vehicle treated littermates. Improvements were significant for declarative memory scores (Morris water maze) and neurological reflexes, as well. Interestingly, delaying the initiation of treatment for 12 h did not diminish the effect of this treatment, and appeared to improve recovery on the beam walk and water maze tasks.

Certain steroids have been shown to benefit the injured brain (Stein and Fulop, 1990). For example, progesterone was found to reduce lipid peroxidation (8-isoprostaglandin-F<sub>2α</sub> levels) in injured brain (Roof et al., 1997). Antiinflammatory glucocorticoids have been shown to down-regulate COX2 expression (Herschman et al., 1995; Masferrer et al., 1992; Yamagata et al., 1993). DHEA steroids have been shown to benefit the aging brain in a number of laboratory studies, and to reduce prostaglandin production in models of peripheral inflammation (Hastings et al., 1988). In cultured primary astrocytes, DHEA (~30 μM) virtually abolished inflammatory-mediated TNF-α and IL-6 production, while not affecting NO or PGE<sub>2</sub> synthesis (Kipper-Galperin et al., 1999). Interestingly, whereas glucocorticoids had their maximal effect when given 18 h prior to mycoplasma or LPS challenge, “DHEA exerted its optimal effect when added together with the activating agents. When added 18 hours prior to activation, it was ineffective” (Kipper-Galperin et al., 1999). This not only indicates a distinct antiinflammatory mechanism from glucocorticoids, but suggests a time dependence for maximal activity that may relate to our *in vivo* results. DHEF was less effective on the beam walk and water maze tasks when the initial dose was administered at 2 h postinjury, compared to earlier or later initial doses (Figs. 2B and 3).

COX2 expression in the brain can be induced by inflammatory cytokines (Moore et al., 1999; Seibert et al., 1994) and by glutamatergic excitation (Hewett et al., 2000; Strauss and Marini, 2002). Increases in patient plasma cytokine levels (IL-1β, IL-6, IL-8, TNF) in the acute response (Ott et al., 1994) to brain injury might be responsible for prolonged COX2 induction in the injured brain. Although DHEF blocked IL-1–induced COX2 in cell cultures, DHEF did not affect injury-induced brain COX2 mRNA levels. Thus, the induction of brain COX2 following TBI may be mediated primarily via excitotoxic rather than inflammatory mechanisms (Strauss et al., 2000). This does not rule out the possibility that DHEF affects IL-1–mediated COX2 expression in other regions.

The DHEA class of steroids inhibit inflammation through pleiotropic mechanisms distinct from both NSAIDs and glucocorticoids. They may act by inhibiting glucose-6-phosphate dehydrogenase (G6PDH) and suppressing oxygen-free radical formation (Babor, 1982; Imlay, 1988; Sadowski, 1985; Schwartz and Pashko, 1993, 1994). DHEA steroids are also very effective inhibitors of NO production. DHEA (10 and 100 μM) reduces mouse macrophage NO production by 50% and 95%, respectively (Mei et al., 1995). DHEF is even more potent; 1 μM DHEF lowers NO production by 60%. DHEF has a much higher affinity for G6PDH than DHEA (K<sub>i</sub> = 0.51 μM and 18.7 μM, respectively; Schwartz et al., 1988). If DHEF improves behavioral recovery via G6PDH inhibition, it should be more potent than DHEA. However, the observation that the *ex vivo* vasoactivity of these two compounds is virtually identical (Fig. 7) indicates a G6PDH-independent mechanism, at least in the cerebrovascular response. DHEA and DHEF may inhibit the responsiveness of the cerebral vasculature to increased concentrations of arachidonic acid metabolites (e.g., produced by phospholipase activation) or free radicals (e.g., produced by COX2 and/or NO synthase).

Treatment with DHEA or related steroids might protect neurons against excitatory amino acid induced damage. *In vivo*, DHEA protected hippocampal CA1/2 neurons against unilateral infusion of NMDA (Kimonides et al., 1998), and reduced CA1 neuronal death in a transient forebrain ischemia model (Li et al., 2001). DHEA and DHEA-sulfate (DHEAs) reduced the neurotoxic actions of NMDA, AMPA, and kainic acid induced toxicity in primary hippocampal cultures (Kimonides et al., 1998). In addition, DHEAs (10 nM) enhanced neuronal and glial survival and differentiation *in vitro* (Bologa et al., 1987; Roberts et al., 1987). These results suggest that DHEAs have a generally protective action against glutamate neurotoxicity. Further studies are underway to characterize the effect of DHEF treatment on injury-induced neuronal survival.

In response to brain injury, reactive astrocytes and microglia proliferate, secrete growth factors and cytokines, and participate in the repair of the disrupted blood–brain barrier. However, excessive proliferation of glia forms a physiological barrier that may impede plasticity and repair (Schumacher et al., 1996). In addition, microglia may contribute to the release of reactive oxygen species, nitric oxide, and glutamate. As a potent G6PDH inhibitor, DHEF may behave as an antioxidant and antiproliferative agent, retarding glial scar formation and enhancing neurite regrowth. Recent studies have shown that the progesterone, 17 $\beta$ -estradiol, testosterone, pregnenolone, and DHEA regulate gliosis in male rat brain (Cerro et al., 1995; García-Estrada et al., 1999). DHEA was the most potent neurosteroid in reducing cellular proliferation and gliotic scar formation in a penetrating brain injury model. However, no alterations in cerebral glial fibrillary acidic protein immunostaining were observed in DHEF-treated injured animals in this study (data not shown).

The GABA-active neurosteroids are modulators of synaptic events and may play a vital role in neuronal plasticity. DHEAs at low micromolar concentrations are allosteric antagonists of the GABA<sub>A</sub> receptors, and non-competitively modulate GABA-induced chloride currents in neurons by accelerating desensitization of GABA<sub>A</sub> receptors (Balieu and Robel, 1996; Imamura and Prasad, 1998; Majewska, 1995; Majewska et al., 1990). DHEAs reduce the amplitudes of GABA receptor-mediated inhibitory postsynaptic potentials (Spivak, 1994), implying a possible enhancement of depolarization in neurons. DHEA injected intraperitoneally in high doses (100–150 mg/kg) produces tonic-clonic seizures in mice (Heuser, 1961; Reddy and Kulkarni, 1998), but at lower doses causes sedation (Majewska et al., 1990). Neither sedation nor seizures were observed in these studies (DHEF, 25 mg/kg). Because different neurosteroids (as well as different doses of a single neurosteroid) modulate GABA<sub>A</sub> receptor function in opposing directions, the *ratio* between excitatory and inhibitory inputs may be the critical factor in reshaping synaptic activity (Majewska, 1995).

Finally, Reddy and Kulkarni (1998) have recently shown that DHEAs facilitated memory in a modified-passive avoidance memory retention task, and suggested a role for central glutamate receptors in the memory modulating effects of neurosteroids. Thus, DHEA and its analogs may counteract some of the effects of injury or aging on neurons, thus promoting neuronal survival and improving memory via multiple mechanisms of action (Flood et al., 1988a,b; Roberts et al., 1987).

Previous studies have demonstrated androgen cerebral vasoactivity in humans and animals. Hata et al. (1996) showed a transient reduction in the pulsatile index (a measure of vascular resistance) of fetal middle cerebral artery in full term women receiving 200 mg intravenous DHEA-sulfate, with no changes in umbilical artery or fetal heart rate (Hata et al., 1996). The direct vasodilatory activity of DHEA and DHEF on bovine middle cerebral artery smooth muscle demonstrates yet another potential avenue for improving recovery, although the mechanism is as yet unknown. While the IC<sub>50</sub> is about four-fold the plasma level achieved with an oral dosage form in rats (200 mg/kg), and within an order of magnitude of those produced in patients (1600 mg/day, p.o.) in a phase I study, several-fold higher peak blood concentrations can be achieved with intraperitoneal administration, as in this study (A. Schwartz, personal communication). Development of a parenteral formulation will be necessary for clinical utility of this class of compounds.

There are no drugs available today that improve the behavioral deficits (changes in affect, memory, coordination, and loss of motor function) suffered by many patients with TBI. In our animal model, DHEF given preor up to 12 h postinjury significantly improved behavioral recovery at 3 days. Possible mechanisms of actions of DHEA and related steroids include attenuation of free radical production via G6PDH inhibition, allosteric modulation of GABA<sub>A</sub> receptors, and inhibition of NMDA, AMPA and kainic acid receptor toxicity via



modulation of central  $\sigma$  receptors. Further work is underway to elucidate which of these mechanisms may contribute to the behavioral improvements seen in our model of TBI. Nevertheless, these results support further development of DHEF in the treatment of TBI.

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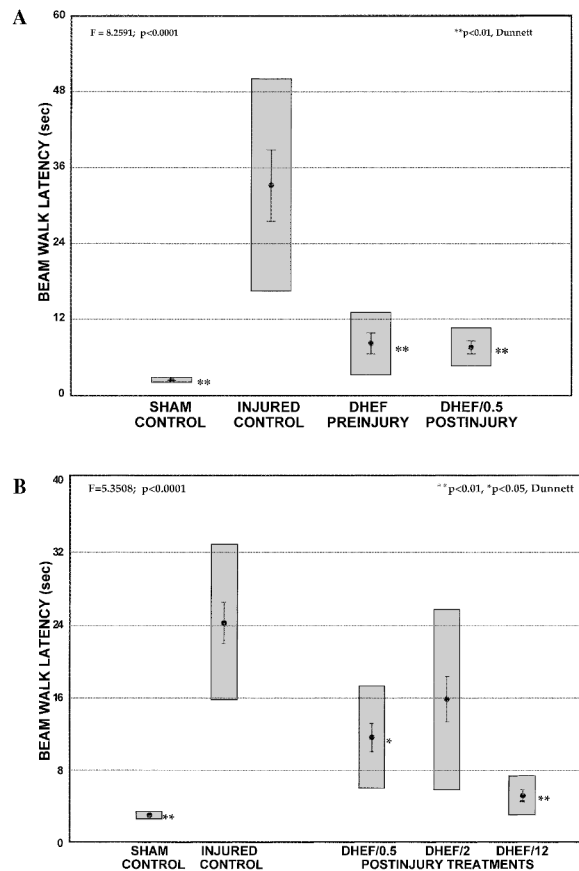
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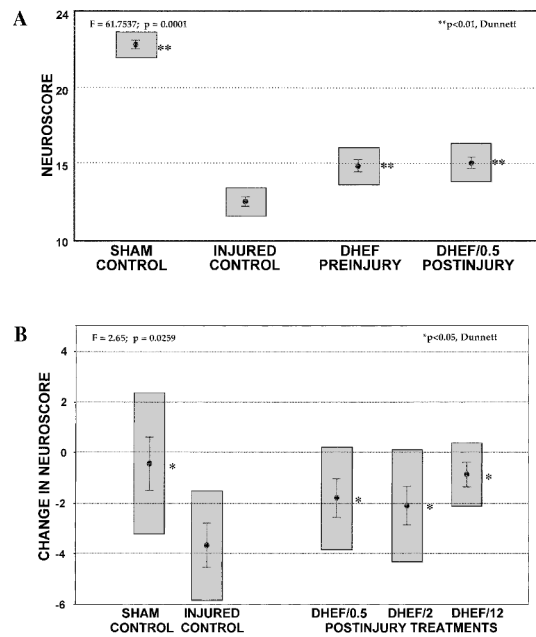
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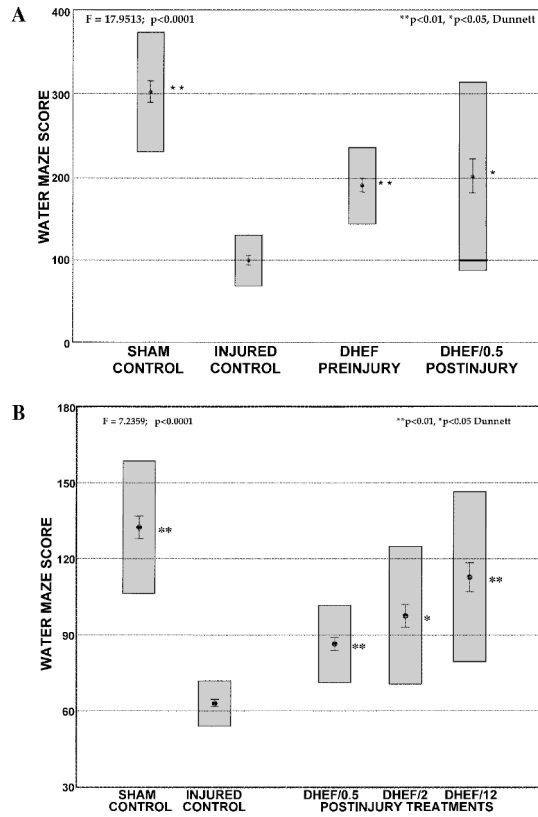
**FIG. 1.** Dehydroepiandrosterone (DHEA) and its analogs function via pleiotropic mechanisms that are independent of androgen and other steroid receptors. These include glucose-6-phosphate dehydrogenase enzyme inhibition, alteration of cytokine-induced gene expression (e.g., attenuation of IL-1 $\beta$ -induced COX2), and allosteric modulation of neurotransmitter receptors (e.g., GABA<sub>A</sub> receptor antagonism).



**FIG. 2.** DHEF treatment improved beam walk performance tested at 3 days after traumatic brain injury. **(A)** Reduced latency to complete the beam walk task in brain injured animals treated before (DHEF preinjury) or one half hour after (DHEF/0.5 postinjury) TBI, as compared to injured animals treated with vehicle. **(B)** DHEF treatments initiated 0.5 h (DHEF/0.5) and 12 h (DHEF/12) after TBI reduce the mean latency to complete the beam walk 3 days postinjury. Sham animals were surgicized but not injured. Results are presented as mean  $\pm$  SEM, gray boxes represent the 99% confidence interval. \* $p < 0.05$ , \*\* $p < 0.01$ , Dunnett versus injured controls.

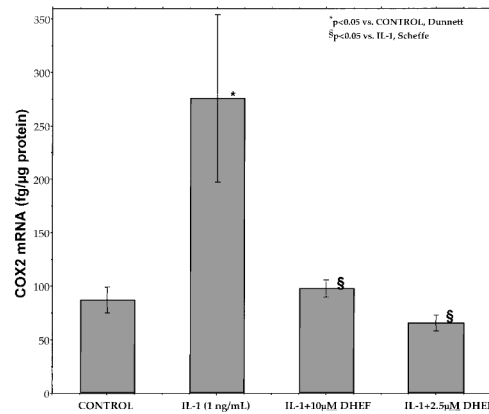


**FIG. 3.** DHEF treatments improved neurological reflexes tested 3 days postinjury. **(A)** Improved recovery of neurological reflexes in injured animals treated before or one half hour after TBI, compared to vehicle-treated injured controls. Neuroscores are presented as mean  $\pm$  SEM. **(B)** Posttreatment with DHEF (0.5, 2, and 12 h postinjury) revealed a small but statistically significant improvement in neurological reflexes. Results are mean difference (postinjury – preinjury)  $\pm$  SEM. Gray boxes represent the 99% confidence interval. \* $p < 0.05$ , \*\* $p < 0.01$ , Dunnett versus injured controls.



**FIG. 4.** DHEF improves declarative memory function tested 3 days postinjury. **(A)** Improvements were observed in Morris water maze performance in brain-injured rats treated with DHEF (10 min before, 0.5 h after injury) compared to vehicle-treated injured controls 3 days postinjury. **(B)** Posttreatment with DHEF (0.5, 2, and 12 h postinjury) also revealed statistically significant improvements in memory function. Water maze score calculated as described in Methods. Results presented are mean  $\pm$  SEM; gray boxes represent the 99% confidence interval.  $**p < 0.01$ ,  $*p < 0.05$ , Dunnett versus injured controls.

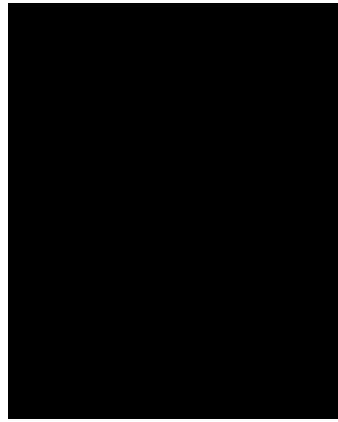


**FIG. 5.**

DHEF attenuates the induction of COX2 mRNA by interleukin-1 $\beta$  in rat mesangial cells. The rat mesangial cell model of *in vitro* inflammation normally shows a robust induction of COX2 gene expression following a 4-h exposure to IL-1 $\beta$  in the absence of serum. DHEF (2.5 and 10  $\mu$ M) completely blocks this, indicating a role for DHEA steroids in restricting inflammatory gene expression. Cell cultures and mRNA measurements performed as described in Methods. Results are mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$ , Dunnett versus control; § $p < 0.05$ , Scheffé versus IL-1 group.



**FIG. 6.** DHEF does not alter COX2 mRNA levels in injured cortex and hippocampus after lateral cortical impact TBI. Rats were injured and sacrificed at various times after TBI. Fresh frozen brain tissue was sectioned (300  $\mu\text{m}$ ) and micropunch ribonuclease protection assays were performed on cortex and hippocampus ipsilateral (and contralateral, not shown) to injury. No inhibition of COX2 induction was observed in these brain areas. Sham controls from multiple time points were grouped (SHAMS). Dashed lines represent injured DHEF pretreatment; solid lines are injured controls. Results are mean  $\pm$  SEM ( $n = 4-6$  per group). \* $p < 0.05$ , Dunnett versus SHAMS.



**FIG. 7.**  
DHEF and DHEA demonstrate vasodilatory effects on bovine middle cerebral artery. Serial addition of DHEA or DHEF to  $K^+$  constricted bovine middle cerebral artery preparation (bMCA) causes vasodilatation with  $IC_{50} \approx 40 \mu M$ .

**Table 1**  
**DHEF TREATMENTS GROUPS: ADDITIONAL WEIGHT LOSS AND IMPROVED BEAM WALK PERFORMANCE AT 3 DAYS POSTINJURY**

Group name	Study (n)	Treatment <sup>a</sup>	Description	Weight loss (g)	Falls from the beam (n)
Sham	A (22)	Sham/vehicle	Surgery, no injury, vehicle 10 min prior	0.1 ± 1.9	n.d.
Injured	A (30)	Injured/vehicle	LCI injury, vehicle 10 min prior	5.8 ± 1.4 *	n.d.
DHEF	A (24)	Injured/DHEF	DHEF started 10 min before LCI injury	15.2 ± 2.3 *§	n.d.
DHEF/0.5	A (15)	Injured/DHEF/0.5	LCI, DHEF started 0.5 h after LCI injury	14.5 ± 4.5 *§	n.d.
Sham	B (17)	Sham/vehicle	Surgery, no injury, vehicle 10 min prior	0.7 ± 2.2	0
Injured	B (26)	Injured/vehicle	LCI injury with vehicle	7.7 ± 2.1 *	9
DHEF/0.5	B (24)	Injured/DHEF/0.5	LCI, DHEF started 0.5 h after LCI injury	17.8 ± 2.8 *§	1 †
DHEF/2	B (23)	Injured/DHEF/2	LCI, DHEF started 2 h after LCI injury	15.8 ± 1.4 *§	4
DHEF/12	B (16)	Injured/DHEF/12	LCI, DHEF started 12 h after LCI injury	17.1 ± 3.1 *§	1 †

n.d., not done.

<sup>a</sup> All groups received initial intraperitoneal injections of vehicle or DHEF (25 mg/kg) at the indicated time, then at 24 h and 48 h postinjury.

\*  $p < 0.05$ , Dunnett versus sham

§  $p < 0.05$ , Scheffé versus injured/vehicle

†  $p < 0.05$ ,  $\chi^2$  analysis.