Treatment of Head Injury in Mice, Using a Fructose 1,6-Diphosphate and Dimethyl Sulfoxide Combination

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MICE WERE SUBJECTED to moderate (800 g/cm force) or severe (900 g/cm force) head injury and treated 5 minutes later with various compounds. Treatments consisted of intravenous administration of the following compounds: 1) fructose 1,6-diphosphate (FDP), 2) dimethyl sulfoxide (DMSO), 3) FDP + DMSO, and 4) vehicle nontreated. Sensory-motor evaluations 1 and 2 hours after 800 g/cm-force head injury showed that significant protection of motor function (grip test) was achieved with FDP + DMSO but not with treatment by either drug alone. Evaluation of mice following a severe 900-g/cm force injury demonstrated significant survival after treatment with FDP + DMSO but not with the vehicle or treatment by either of these agents alone. Histopathological morphometry indicated that cortical and hippocampal CA1 neurons were markedly protected from damage when mice were treated with combined FDP + DMSO. More modest protection of CA1 but not of cortical neurons was observed after treatment with DMSO alone but not after treatment with FDP alone or administration of the vehicle. These findings indicate that combining FDP with DMSO results in considerable synergy in protecting animals from sensory-motor loss and neuronal brain damage and in ultimate survival stemming from a moderate or severe closed head injury. (Neurosurgery 37:273–279, 1995)

Key words: ATP head injury, CA1 hippocampus, Dimethyl sulfoxide, Free radicals, Fructose 1,6-diphosphate, Neuronal damage

Brain trauma caused by severe blunt injury to the head often results in a grim prognosis because of the lack of an effective treatment that can reverse the physiopathological cascade associated with this injury. The poor prognosis associated with serious head injuries in patients is often the result of complications leading to the development of progressive intracranial pressure increase, ischemia, and coagulopathy (5, 17, 38, 41). Prolonged cerebral edema and ischemia can result in extensive neuronal damage and irreversible sensory-motor deficits (5).

The glycolytic intermediate fructose 1,6-diphosphate (FDP) and the free radical scavenger dimethyl sulfoxide (DMSO) have each been reported to be useful when administered in head-injured (10, 14, 15, 23, 40) or cerebral ischemic (12, 16, 28, 34) animals or humans. FDP has been shown to prevent ischemic-induced loss of adenosine triphosphate (ATP) and accumulation of intracellular Ca^{2+} in experimental animals, possibly by stimulating glycolysis while inhibiting gluconeogenesis (28). Administration of FDP during brain ischemia is particularly useful, because it readily crosses the blood-brain barrier (16) and can yield twice as many moles of ATP as glucose (28). DMSO has been shown to reduce intracranial pressure after head trauma (10, 14, 15, 22, 23, 40) in animals or humans and to increase cerebral blood flow in the presense of brain ischemia (8, 11, 12, 34). DMSO may achieve its beneficial effects in part by its ability to scavenge free radicals (35, 44) and protect cell membranes from physical and chemical injury (1, 11, 35, 42).

Because brain injury can lead to an impairment of mitochondrial oxidative phosphorylation with loss of ATP (37) and to the formation of cytotoxic free radicals (6, 22), we reasoned that a combination FDP + DMSO solution might be more useful than either drug alone in preventing the neuronal and energy compromise associated with severe, concussive head injury in a mouse model.

MATERIALS AND METHODS

Male CD-1 mice weighing 22 to 29 g were restrained, and their heads were positioned under a head injury apparatus with their chins resting firmly on a flat surface at the base of the apparatus. The apparatus consisted of a 25-cm-long glass shaft held vertically by clamps on a ring stand and a tubular lead cylinder weighing 40 g. The lead cylinder was allowed to drop 20 or 22.5 cm through the shaft onto the center of the head, thus creating an 800- or a 900-g/cm force injury on the cranium. An imaginary line was drawn coronally just anterior to the ears in order to create a consistent injury site on the animal's head. This head injury mouse model has been pre-

viously used to screen the effectiveness of potential pharmacological agents after brain trauma (18). The lead cylinder used to induce head injury measured 3 cm in length and had a flat striking surface 5.3 mm in diameter.

Both 800- and 900-g/cm injury results in momentary loss of consciousness and brief seizure activity lasting 1 to 2 minutes. Further seizure activity was not observed in any mouse until death. Mice subjected to 800-g/cm injury were divided randomly into five groups (n = 8/group) as follows: the FDP + DMSO group, the FDP group, the DMSO group, and the vehicle (5% dextrose) nontreated group. An intact (no injury) group was used for reference. All drugs were dissolved in 5% dextrose in water solution. FDP was given at 350 mg/kg in a 10% solution, and DMSO was administered at 1 gm/kg in a 28% solution. When combined, FDP and DMSO retained the same final concentrations as when used alone.

The crystalline trisodium salt of FDP (Sigma, St. Louis, Missouri) was first dissolved in sterile 5% dextrose in water by vigorous agitation and was then added to the DMSO solution (FDP is not directly soluble in DMSO) or injected alone. DMSO (Pharma 21, Portland, Oregon) was used as 99.9% pharmaceutical grade, sterile, pyrogen-free solution.

Drugs were injected at volumes ranging from 0.07 to 0.08 ml via the dorsal tail vein 5 minutes after trauma. The surviving mice were then returned to a heated cage, where body temperature was maintained, and 1 and 2 hours after head trauma, each mouse was tested neurologically for sensory-motor deficits.

Sensory function was evaluated by lightly squeezing the first finger or toe bilaterally with tweezers. Sensory deficit was noted if no retraction of the finger/toe was elicited after light squeezing (9). Motor function was evaluated by grasping each mouse by the tail to allow the front or rear paws to come in contact with the center of a taut, 60-cm string stretched between two ring stands and suspended 35 cm from a padded table below (grip test). Upon contact with the string, the tail was released and the length of time the mouse remained grasping the string was measured in seconds (18). Any mouse that remained grasping the string for 90 seconds was removed from the string and returned to its cage.

All mice were pretested on the string prior to head injury, and all were able to grasp the string for a 90-second trial period and to travel with ease from the center of the string to one of the end posts holding the string. The extent of motor deficits reflected by the reduced ability of mice to remain gripping the string after head injury has been shown to be inversely proportional to the increased severity of the head injury (39).

Each mouse was placed only once on the string for each of the two post-trauma test periods. An additional test was used to measure the relative strength of the fore and hind paws by noting whether the mouse was able to travel from the center of the string to one of the end posts, a task requiring good motor function of all four limbs (18).

A second group of mice (n = 6) was subjected to a more severe 900-g/cm injury using the same doses, treatment times, drugs, and route of administration. Any mouse surviving this injury was given a "grip test" after 1 hour.

All mice were killed by decapitation 3 hours after the initial head injury, and the brains were processed for Palmgren silver impregnation stain. Four-micron coronal sections were taken every 100 μm, beginning at the center of the lesion site and proceeding in an anteroposterior direction from site of trauma. Fixation artifacts did not appear to have developed in the brain sections examined, as evidenced by the absence of damaged neurons (identified as pyknotic and hyperchromatic [7]) in the intact control group as well as the reduced number of injured neurons in the drug-treated groups. To assess neuronal damage, dorsal CA1 hippocampal sector and frontoparietal cortex were used for morphometry. The hippocampal region was chosen for morphometry even though it was not directly below the site of trauma, because this region is highly vulnerable to ischemia and to cerebral swelling and also because the hippocampus has been linked to learning and memory, functions which are often affected after severe brain trauma (26). Frontoparietal cortical neurons were evaluated because of their proximity to the lesion site and because damage to this region was relatively consistent among the experimental and nontreated groups. Neuronal counts of the frontoparietal cortex and CA1 region were made by averaging the number of damaged per total neurons from each side of the hippocampus or parietal cortex from five representative sections. All cell counts were made by an observer who was blind to the treatment groups. Group means were obtained for each treatment, and these values were compared with vehicle nontreated controls. Morphometric data was analyzed for specific intergroup comparisons, using a digitizing screen connected to a Jandel program that computed means, sum, standard deviation, and standard error, using the Student's *t* test. Statistical analyses of grip test scores for each time point were made using one-way analysis of variance and post hoc Bonferroni correction for multiple comparisons of mean grip times of vehicle nontreated mice to each experimental group treated with single doses of FDP, DMSO, or combined FDP + DMSO and between all possible combinations of the groups. The software program, Instat v.2, was used for the calculations. A value of $P < 0.05$ was considered significant.

RESULTS

An examination of the brains after removal from the calvarium indicated that a subdural or subarachnoid hemorrhage on the side of the lesion was present in all groups regardless of treatment. No intraparenchymal hemorrhages were seen on inspection of the brain slices in any animal.

Figures 1 and *2* show the relative effects of drug treatment on the protection of the frontoparietal cortex and CA1 in mice subjected to 800-g/cm concussive head injury. Morphometry of neurons in CA1 and cortex was not attempted after 900-g/cm injury (see *Table 2*). *Figures 1* and *2* summarize the morphometric neuronal counts in the two brain regions examined (frontoparietal cortex and CA1 hippocampus).

Histological analysis of frontoparietal cortical neurons at the site of injury showed marked necrosis and severe damage of neurons in vehicle nontreated mice as compared with the intact no-injury group (*Fig. 3, B, D, F, H,* and *J*). The area of

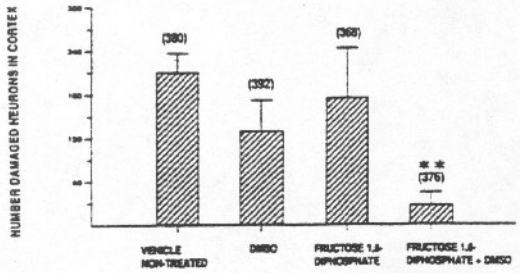

FIGURE 1. Treatment efficacy in the cortex after an experimental moderate head injury (800 g/cm). This bar graph shows the mean number of damaged neurons in mouse frontoparietal cortex after 800-g/cm head injury and treatment with DMSO, FDP, or FDP + DMSO, as compared with the vehicle nontreated group. The mean total number of intact neurons is shown in *parentheses* above the *bars*. The number of damaged neurons after FDP + DMSO treatment is significantly lower than the number of damaged neurons in the vehicle nontreated group. *Bars* indicate the standard error of the mean. **, $P < 0.001$ versus the results for the vehicle nontreatment group.

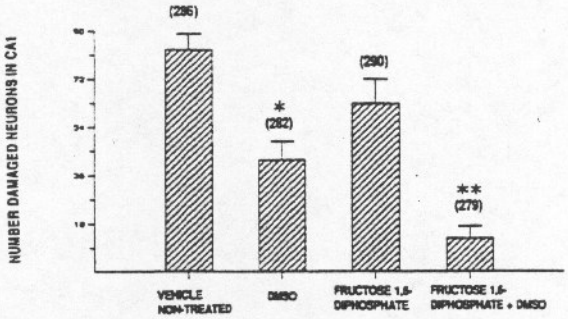

FIGURE 2. Treatment efficacy in the CA1 hippocampus after an experimental moderate head injury (800 g/cm). This bar graph shows the mean number of damaged neurons in the mouse hippocampus after 800-g/cm head injury and treatment with DMSO, FDP, or FDP + DMSO, as compared with the vehicle nontreated group. The mean total number of intact neurons is shown in *parentheses* above the *bars*. The number of damaged neurons after FDP + DMSO treatment is significantly lower than the number of damaged neurons in the vehicle nontreated group. *Bars* indicate the standard error of the mean. *$P < 0.01$; **, $P < 0.001$ versus the results for the vehicle nontreatment group.

cortical damage extended several hundred microns from the site of injury in an anteroposterior direction and often included subcortical areas in the preoptic region, medial septum, and striatum. Mild to moderate disruption of the myelinated fibers in the caudate nucleus was a common histopathological finding in mice subjected to 800-g/cm head injury, but no quantification of this damage was attempted. Neuronal protection of the frontoparietal cortex was significant only in the FDP + DMSO-treated group (*Fig. 1*). The density of perineuronal spaces (indicating loss of neurons) was highest in the vehicle nontreated mice (averaging five or more spaces/high-powered field), moderate in FDP (three/high-powered field), and mild (less than three/high-powered field) in the DMSO and FDP + DMSO groups (*Fig. 3, D, F, H,* and *J*). CA1 neuronal damage was more severe in the external (superior) row of neurons, with middle row neurons incurring moderate damage and the internal (inferior) blade of neurons showing the least amount of injury (*Fig. 3, E, G,* and *I*). The density of perineuronal spaces in CA1 was highest in vehicle nontreated animals (*Fig. 3J*) and was mild in all the experimentally treated groups (*Fig. 3, C, E,* and *G*). Scarce or no perineuronal spaces were observed in the FDP + DMSO group in the CA1 region (*Fig. 3C*).

Sensory function was present in 11 of 12 mice treated with FDP + DMSO, as compared with 5 of 12 mice given vehicle nontreatment, 8 of 12 treated with DMSO, and 7 of 12 treated with FDP. The combination of FDP + DMSO significantly protected mice from motor deficits after injury, as indicated by an increased mean grip test score (76.3 s) shown by this group 1 hour after head injury when compared with vehicle nontreated (5.8 s), FDP-treated (20.8 s), or DMSO-treated (19.6 s) animals (*Table 1*). *Table 1* demonstrates that the pattern of protection continued for at least 2 hours after head injury, with all groups showing some improvement over the previous grip test taken 1 hour after head injury. However, the only significant protection after 2 hours was observed in the group treated with FDP + DMSO. Two hours after head injury, the group treated with FDP + DMSO had a mean grip test score of 86.5 seconds, which was significantly different from the DMSO (33.8 s), FDP (24.3 s), and vehicle nontreated groups (12.2 s).

When the severity of head injury was increased to a 900-g/cm force, a marked increase in mortality resulted in vehicle nontreated, FDP, and DMSO groups (*Table 2*). By contrast, mortality was reduced significantly in the FDP + DMSO group. Moreover, among the survivors of this severe injury, mean grip test times were significantly higher in the FDP + DMSO (26 s) when compared with the FDP (2 s) or DMSO (6 s) groups; vehicle nontreated animals were not scored, because no animal from this group survived the 900-g/cm severe head trauma (*Table 2*).

DISCUSSION

The present study is the first to demonstrate that the combined FDP + DMSO given to mice that have been subjected to moderate (800 g/cm) or severe (900 g/cm) concussion head injury can significantly modify the sensory-motor deficits, incidence of mortality, and histopathology. FDP + DMSO appeared superior in reducing the trauma-induced neurological deficits and fatal outcome to either agent administered alone.

FDP is a versatile compound that has been reported to improve neurological recovery in rabbits after resuscitation from cardiac arrest (16) and to reduce infarct volume after middle cerebral artery occlusion in rats (25). FDP has been shown to protect the brain from ischemic-hypoxic injury, although the exact mechanism of how this is achieved remains speculative (16).

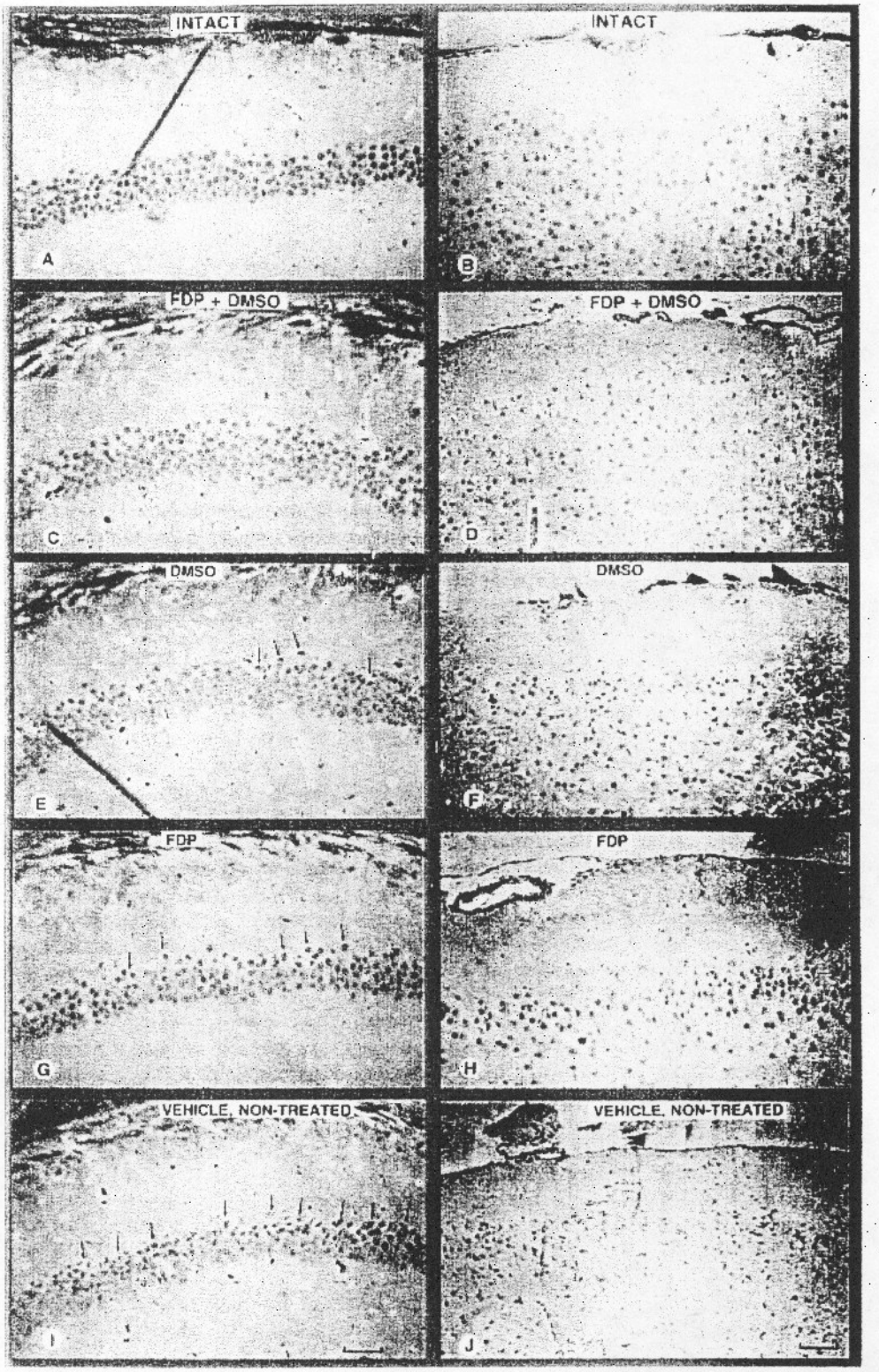

FIGURE 3. Representative silver-stained sections of mouse CA1 hippocampus (*A, C, E, G,* and *I*) and frontoparietal cortex (*B, D, F, H,* and *J*) after 800-g/cm head injury with subsequent treatment with FDP, DMSO, FDP + DMSO, and 5% dextrose in water (vehicle nontreated). Note the normal shape and staining color of the CA1 and cortical neurons in noninjured control mice (*A* and *B*) and in mice treated with FDP + DMSO (*C* and *D*). By contrast, moderate neuronal damage (*arrows* indicate pyknotic, hyperchromatic neurons) in CA1 hippocampus and severe damage in cortex (characterized by shrunken neurons often surrounded by periaxonal spaces) is seen after treatment with FDP alone (*E* and *F*) and DMSO alone (*G* and *H*). Severe neuronal damage is noted in the vehicle nontreatment group, in which injury extends to practically all of the external row of neurons of the CA1 hippocampus (*I*) and in the cortex (*J*), where many perineuronal spaces are associated with shrunken, hyperchromatic neurons, especially in cortical layers III and V. *Bar,* 200 μm.

TABLE 1. Mean Grip Time on String after Moderate Head Injury[a]

Group	Mean Grip Time (s)	
	Grip Test 1 hr after Trauma	Grip Test 2 hr after Trauma
Vehicle nontreated	5.8 ± 2	12.2 ± 4
DMSO	19.6 ± 6	33.8 ± 8
FDP	20.8 ± 4	24.3 ± 6
FDP + DMSO	76.3 ± 9[b]	86.5 ± 7[b]
Intact controls	90 ± SEM	90

[a] Mean scores in seconds (±the standard error of the mean [SEM]) in mice holding on to a string (grip test) 1 or 2 hours after moderate (800 g/cm) head injury. FDP, fructose 1,6-diphosphate; DMSO, dimethyl sulfoxide; vehicle nontreated, 5% dextrose in water; intact controls, no trauma. The FDP + DMSO group showed significant protection from motor deficit, which improved slightly from 1 to 2 hours after injury. One-way analysis of variance compared all groups to intact controls.
[b] $P < 0.001$.

TABLE 2. Mean Grip Time and Survival after Severe Head Injury[a]

Group	Number of Mice (Dead/Surviving)	Mean Grip Time (s)
FDP + DMSO	1/5[b]	26.4 ± 3[b]
FDP	2/4	2.8 ± 0.6
DMSO	2/4	6.3 ± 0.9
Vehicle nontreated	6/0	—

[a] Mean group scores (±the standard error of the mean) of mice holding on to a string (grip test) after severe (900 g/cm) head injury was influenced by the high mortality in all groups except for the FDP + DMSO Group. FDP, fructose 1,6-diphosphate; DMSO, dimethyl sulfoxide; vehicle nontreated, 5% dextrose in water; —, no mean grip time was available for the vehicle group because none of those mice survived the severe injury.
[b] $P < 0.01$.

Brain ischemia is known to cause rapid depletion of ATP stores, a condition that eventually leads to failure of aerobic glycolysis (37). The compromised aerobic metabolism forces a shift to the less energy-effective anaerobic glycolysis, which in turn increases lactate levels. Increased lactate results in acidosis, which can inactivate the pH-sensitive, rate-limiting enzyme phosphofructokinase (PFK) (24), a condition that further supresses aerobic glycolysis (37). As cerebral energy supply declines, cellular depolarization and intraionic homeostasis is upset, resulting in intracellular K^+ efflux and Ca^{2+} influx, a phenomenon that causes additional damage to nerve cells (19, 37). It is logical to assume that if cerebral ischemia or hypoperfusion is present, correcting the acidotic state or bypassing the suppressed PFK enzyme step would restore aerobic glycolysis and energy metabolism. This latter, in fact, can be achieved by administering FDP, a natural high-energy intermediate in the glycolytic pathway that supplies high-energy phosphates to tissue during hypoxemic-ischemic episodes (24). Because oxidative phosphorylation and the manufacturing of ATP is depressed during ischemia, FDP cannot be normally phosphorylated in brain. Moreover, the levels of FDP are reduced during ischemic-induced lactic acidosis because, as stated above, PFK is inhibited by lactic acid production (16). As high-energy phosphate supply to the brain declines, respiratory, cardiac, and cerebral metabolism becomes critically compromised. Administration of FDP during hypoxia-ischemia, even in the presence of lactic acidosis, can theoretically bypass the PFK conversion to FDP and thus increase ATP production and glycolysis (28, 31). An advantage to administering FDP over glucose during brain ischemia is that each mole of FDP yields 4 mol of ATP, whereas glucose yields only 2 mol of ATP (32), because glucose requires phosphorylation at two steps that expend 2 mol of ATP. Another advantage to using FDP after brain injury may be its ability to reduce oxygen radicals generated by stimulated neutrophils (29). This situation can occur when blood flow is re-established in previously ischemic tissue (36).

DMSO is an agent with a wide range of biological activities (10, 11, 35, 42–44). For example, DMSO is reported to protect cell membranes (1, 42), improve central nervous system blood flow after injury (7, 12, 15, 34), reduce intracranial pressure after experimental (15) or clinical head trauma (23, 40), and powerfully scavenge hydroxyl radical formation (35, 44).

The combined activity of FDP + DMSO after head trauma may work to prevent or restore loss of ATP in ischemic brain cells while simultaneously reducing progressive cerebral edema, which can generate further cerebral ischemia. Thus, FDP would act primarily to protect ischemic brain tissue from energy substrate depletion and DMSO would act to stabilize cell membranes from possible attack by hydroxyl radicals while reducing intracranial hypertension and improving cerebral blood flow. Both drugs would thus address key pathogenic elements generally observed after head injury better than each agent used alone. FDP + DMSO treatment may therefore correct possible energy substrate reduction, abnormal Ca^{2+} entry and accumulation in cells, hydroxyl radical formation, increased intracranial pressure, and cerebral blood flow decline (13).

Another possible "fringe benefit" from FDP + DMSO therapy that could increase protection to the head-injured brain from inadequate cerebral perfusion may be the ability of these compounds to allow red blood cells (RBCs) to maintain their deformability. Under normal conditions, RBCs with a 7- to 8-μm diameter can squeeze through capillaries that are 3 to 5 μm wide so that oxygen can be delivered to the tissues. The RBCs' viscoelastic properties or deformability are possible because of a spectrin-actin interaction within the erythrocyte, which is dependent on its stored ATP and 2,3-diphosphoglycerate (27). If 2,3-diphosphoglycerate or ATP stores are reduced by ischemia-hypoxia (20, 27), it is probable that RBCs will increase their rigidity and thus be unable to adequately perfuse the cerebral microvasculature. The consequence would prevent optimal oxygen delivery to neurons and glial cells, which rely on oxygen for elaboration of ATP and for their basic cellular metabolism. Infusion of FDP raises the concentration of 2,3-diphosphoglycerate (20) allowing RBCs to deform normally and consequently facilitating oxygen disassociation from hemoglobin (3, 4) during ischemia-hypoxia.

Conversely, DMSO is reported to promote the formation of ATP from ADP (2) and to preserve mitochondrial oxidative phosphorylation, thus ensuring ATP production after experimental brain ischemia (14). This activity could additionally favor RBC deformability during tissue ischemic-hypoxia.

The side effects that have been clinically observed with high doses of intravenous DMSO are red cell lysis when used in concentrations above 40% (40) and hypernatremia with fluid overload when used in concentrations of 10% or lower (33). These side effects are not observed when DMSO is used at a concentration of 28%. Clinical side effects of FDP in patients with acute myocardial infarction include an increase in plasma inorganic phosphate and a reduction in cholesterol and triglycerides as well as a decrease in heart rate and pulmonary artery pressure (30). These values return to normal levels following discontinuation of FDP.

Our findings indicate that FDP combined with DMSO can significantly reduce motor deficits, improve survival, and protect neuronal tissue from the histopathological changes that are encountered after severe blunt head injury in mice. The reason for the synergic activity demonstrated by the FDP + DMSO combination may lie in the ability of each agent to address different but critical metabolic abnormalities associated with the pathogenesis of severe head injury (e.g., ATP loss, acidosis, abnormal Ca^{2+} entry into cells, intracranial pressure increase, and free radical formation). We are presently studying the effects of this drug combination after experimental head trauma to determine whether any secondary injury occurs when the observation period is extended beyond 3 hours.

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COMMENTS

This report indicates that dimethyl sulfoxide (DMSO) and fructose 1,6-diphosphate (FDP) have a synergistic protective effect in a mouse model of moderate and severe head injury. Despite optimistic reports on the benefits of DMSO in the laboratory setting, there has been limited enthusiasm for this agent after early clinical trials.

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The author reports that a combination of two drugs, FDP and DMSO, reduces brain damage compared with vehicle controls. Other investigators have shown that DMSO or FDP alone can have neuroprotective effects, but this study suggests that neither drug alone conferred significant neuroprotection. Theoretically, these two drugs should have different mechanisms of action. FDP is a phosphorylated carbohydrate that presumably acts by contributing energy substrate, and DMSO is a solvent that can scavenge damaging hydroxyl free radicals. The study does not clarify how or why this combination of drugs is effective although neither drug alone appears to be effective.

The article stresses some of the more interesting explanations of the results but neglects several mundane possibilities. For example, both drugs were given in very high doses and in relatively concentrated solutions. The dose of FDP was 350 mg/kg in a highly concentrated 10% solution plus 5% dextrose. The DMSO dose was even higher, i.e., 1000 mg/kg in a 28% solution plus 5% dextrose. Such hypertonic solutions may well cause diuresis and have osmotic effects similar to therapeutic doses of mannitol, i.e., 1–2 g/kg, used to treat head injury. The control group received only 5% dextrose solutions. These solutions may alter blood pressure, respiratory parameters, body temperature, and other systemic variables known to influence head injury.

The study unfortunately omitted relatively simple measurements that could have ruled out some of these possibilities. For example, monitoring temperature, blood pressure, and blood gases could have eliminated some of the more mundane possibilities. A simple measurement of brain adenosine triphosphate levels in the three treatment groups would have provided strong support for the hypothesis that FDP has effectively contributed metabolic substrates. Finally, if the authors had waited 24 hours before assessing the neuronal morphology, the argument for neuroprotection would have been stronger. Thus, additional studies are needed to confirm the results. Nevertheless, the results are interesting and should stimulate researchers to try these treatments in a more rigorous setting.

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