Dodecafluoropentane Emulsion Decreases Infarct Volume in a Rabbit Ischemic Stroke Model

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ABSTRACT

Purpose: To assess the efficacy of dodecafluoropentane emulsion (DDFPe), a nanodroplet emulsion with significant oxygen transport potential, in decreasing infarct volume in an insoluble-emboli rabbit stroke model.

Materials And Methods: New Zealand White rabbits (N = 64; weight, 5.1 ± 0.50 kg) underwent angiography and received embolic spheres in occluded internal carotid artery branches. Rabbits were randomly assigned to groups in 4-hour and 7-hour studies. Four-hour groups included control (n = 7, embolized without treatment) and DDFPe treatment 30 minutes before stroke (n = 7), at stroke onset (n = 8), and 30 minutes (n = 5), 1 hour (n = 7), 2 hours (n = 5), or 3 hours after stroke (n = 6). Seven-hour groups included control (n = 6) and DDFPe at 1 hour (n = 8) and 6 hours after stroke (n = 5). DDFPe dose was a 2% weight/volume intravenous injection (0.6 mL/kg) repeated every 90 minutes as time allowed. After euthanasia, infarct volume was determined by vital stains on brain sections.

Results: At 4 hours, median infarct volume decreased for all DDFPe treatment times (pretreatment, 0.30% [P = .004]; onset, 0.20% [P = .004]; 30 min, 0.35% [P = .009]; 1 h, 0.30% [P = .01]; 2 h, 0.40% [P = .009]; and 3 h, 0.25% [P = .003]) compared with controls (3.20%). At 7 hours, median infarct volume decreased with treatment at 1 hour (0.25%; P = .007) but not at 6 hours (1.4%; P = .49) compared with controls (2.2%).

Conclusions: Intravenous DDFPe in an animal model decreases infarct volumes and protects brain tissue from ischemia, justifying further investigation.

ABBREVIATIONS

ACA = anterior cerebral artery, DDFP = dodecafluoropentane, DDFPe = dodecafluoropentane emulsion, ICA = internal carotid artery, ICH = intracranial hemorrhage, MCA = middle cerebral artery, PFC = perfluorocarbon, TPA = tissue plasminogen activator

Many diverse situations involving blood loss, ischemia, or hypoxia result in organ and tissue damage that cause morbidity and mortality. These situations include common surgical and interventional procedures as well as trauma and natural disease states. These episodes commonly present as myocardial infarctions, as other hypoxic or ischemic syndromes widely distributed throughout the body and extremities, and also as ischemic strokes. Additionally, clinical procedures including surgery and angiography can produce microemboli resulting in silent or subclinical cerebral ischemia (1). Neuroprotective compounds, hyperbaric oxygen, hemoglobin-based blood substitutes, other approaches, and...
liquid perfluorocarbon (PFC)-based oxygen carriers have shown promise but largely failed to compensate in these situations (2–7). Prompt revascularization and restoration of oxygenated blood flow remain the primary foci of clinical stroke therapy at the present time.

Another oxygen transport substance may have therapeutic potential: because of the highly electrophilic fluorine content and lack of intermolecular attractive forces inherent to PFCs, PFC emulsions have the ability to physically dissolve, transport, and deliver significant quantities of oxygen and other electron-rich respiratory gases (8,9). Sophisticated techniques allow the production of stable PFC emulsions with exceptionally small particles. Such a small-scale droplet allows passage beyond many vascular occlusions that block 8-μm red blood cells, and allows perfusion into even the smallest areas of microcirculation and tissues that would not otherwise be oxygenated by an occluded arterial supply.

Dodecafluoropentane (DDFP) emulsion (DDFPe) is a stable emulsion of 250-nm droplets that, on in vitro administration at 37°C, undergoes expansion into the gaseous state (10,11). This expansion is unique to DDFP among PFCs. DDFP has a boiling point of approximately 29°C; thus, at 37°C, large intermolecular “pockets” open up in the DDFPe droplets, such that high concentrations of respiratory gases can be rapidly drawn within. In vitro, the DDFP droplets eventually expand to form microbubbles. However, in vivo, when DDFPe is injected intravenously, it does not expand to true bubble form (11). The intravascular pressure retards full bubble expansion, but fortuitously allows alternation of droplet swelling and contraction as necessary to absorb and release respiratory gases as the droplets travel through the bloodstream without reaching microbubble size. Liquid PFCs do not possess this ability, which renders them relatively limited in their gas-solubilizing abilities. An in vitro comparison of three PFC emulsions demonstrated markedly superior oxygen delivery for DDFPe in the gaseous state (11). In vivo, DDFPe functions for approximately 2 hours, and the DDFP is exhaled through normal respiration without long-term retention in the body (12).

Here we test this intravenous emulsion therapy in a rabbit model of acute ischemic stroke caused by permanent angiographic occlusions of branches of the internal carotid artery (ICA). The aim is to determine if neuroprotection can be provided without restoration of blood flow.

**MATERIALS AND METHODS**

All animal procedures were approved by the institutional animal care and use committee. New Zealand White rabbits (N = 95 total) were used in this study. Surgical and angiographic procedures were described previously (13,14). Briefly, rabbits were sedated with intramuscular injection of ketamine 30 mg/kg (Ketaset; Fort Dodge, Fort Dodge, Iowa) and xylazine 3 mg/kg (AnaSed; Lloyd Laboratories, Shenandoah, Iowa) and anesthetized with isoflurane (Novaplus; Hospira, Lake Forest, Illinois). A femoral artery was surgically exposed, and a modified 65-cm angled-tip 3-F catheter (Slip-Cath; Cook, Bloomington, Indiana) was advanced via standard angiographic techniques to select the ICA.

Subselective magnification angiography was performed before embolization and 1 minute after embolization to document the precise occlusion of the cerebral vasculature (Fig 1). Imaging was performed by using a single-plane C-arm digital mobile imaging system (OEC 9800; GE Healthcare; Salt Lake City, Utah). Embolization with two or three individual microspheres 700–900 μm in diameter (Embosphere; BioSphere, Rockland, Massachusetts) flushed into the ICA occluded some branches, usually the middle cerebral artery (MCA) and/or anterior cerebral artery (ACA). Repeat angiography 1 minute later confirmed vessel occlusion and compromised flow in the ischemic area. To provide uniform deficits, rabbits with other occlusions or angiographic complications (n = 31) were discarded from the study.

Treatments were initiated according to group schedules by using an ear vein catheter access (Instyle-W; Becton Dickinson, Sandy, Utah). Four or 7 hours after embolization, rabbits were euthanized with 1.5 mL of intravenous pentobarbital (Euthasol; Virbac, Fort Worth, Texas).

For treatments, rabbits were randomly assigned to seven groups in the 4-hour study: (i) control, embolized without therapy (n = 7); (ii) pretreatment with DDFPe 30 minutes before embolization (n = 7); (iii) immediate DDFPe (n = 8); (iv) DDFPe at 30 minutes after stroke (n = 5); (v) DDFPe at 1 hour after stroke (n = 7); (vi) DDFPe at 2 hours after stroke (n = 5); and (vii) DDFPe at 3 hours after stroke (n = 6). The administration of therapy was a slow push intravenous dose of DDFPe (2% weight/volume DDFP, 0.6 mL/kg; NuvOx Pharma, Tucson, Arizona) at the designated group time and repeated every 90 minutes as time before euthanasia allowed.

To observe the limit of treatment efficacy, a parallel study was performed with the use of a much delayed treatment compared with another control group. Groups were control rabbits (n = 6), rabbits treated with DDFPe at 1 hour after stroke with additional doses every 90 minutes (n = 8), and rabbits treated with DDFPe starting at 6 hours after stroke (n = 5). These animals were euthanized 7 hours after embolization.

After euthanasia, the brain was harvested, immediately chilled in saline solution, and then sliced coronally at 4.0-mm intervals by using a chilled brain mold (RBM-7000C; ASI Instruments, Warren, Michigan). Brain sections (n = 8) were placed in 1% 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, Missouri) for 45 minutes at 37°C, fixed in 10% formalin, and digitally photographed (Fig 2). Brain size and areas of infarction were measured by using digital analysis (ImageJ software, National Institutes of Health, Bethesda, Maryland) by a technician blinded to treatment groups. Infarct volume was calculated as a percentage of the whole brain.

Fixed brain sections were embedded in paraffin and sectioned at 4 μm. After a standard hematoxylin and eosin stain, sections were analyzed and then scored for intracranial hemorrhage (ICH), defined as extravasations of eryth-
rocytes and fluid into the extracellular space (15). The presence and location of ICH were recorded by a veterinary pathologist blinded to treatment groups.

Treatment with DDFPe was combined into three important groups for analysis: pretreatment 30 minutes before embolization, hyperacute treatment less than 1 hour after symptom onset, and acute therapy 1–3 hours after onset.

Because infarct volumes were not normally distributed, ranks of infarct volume percentages were analyzed with the PROC GLM (ie, Kruskal–Wallis equivalent) function of SAS software (SAS, Cary, North Carolina). Dunnett-adjusted P values were used in comparing each DDFPe group versus controls. Comparisons of 4- and 7-hour control groups, and of treatment groups within the acute and hyperacute treatment subgroups, were made by using the “exact” procedures in the software package StatXact (Cytel, Cambridge, Massachusetts). The incidence of hemorrhage within or outside the stroke area was compared by using the χ² test and Fisher exact test.

RESULTS

Ninety-five rabbits underwent the angiographic procedure; 11 resulted in severe vasospasm of the ICA and 84 rabbits had successful embolization with permanent occlusion of the MCA and/or ACA. Twenty of these also had occlusion of posterior cerebral or superior cerebellar arteries and were discarded from the study, leaving 64 for analysis. All rabbits were successfully maintained at a normal physiologic state of oxygenation and cardiac function throughout the procedure and treatments.

In the 4-hour study (Table 1), median infarct volumes were decreased (P = .001, exact Mann–Whitney test) for all rabbits treated with DDFPe (n = 38) compared with controls (0.30% vs 3.20%). The hyperacute group median (n = 13; Fig 3) was significantly reduced (0.30%) compared with controls (P = .021, Dunnett-adjusted comparison of ranks; unadjusted P = .008). The acute group median (n = 18) was also reduced (0.30%; P = .005, Dunnett-adjusted comparison of ranks; unadjusted P = .002). The individual groups within the hyperacute and acute categories did not differ from each other (P = .54 and P = .92, respectively, exact Kruskal–Wallis test).

In the 7-hour study (Table 2), control infarct volumes were similar to the 4-hour controls, with a mean of 3.88% and a median of 2.2% (P = .70, exact Mann–Whitney test). The hour-1 therapy animals had seven of eight values at or below the lowest control value, and the hour-6 therapy animals had three of five at or below the lowest control value. Microscopic hemorrhage rates were similar in all groups (n = 44) in the 4-hour study, both in the stroke area (P = .85) and outside the stroke area (P = .32). Hemorrhage within the stroke was seen in 14% of control animals (n = 1 of 7), 14% of the DDFPe pretreatment group (n = 1 of 7), 14% of the immediate DDFPe group (n = 1 of 7),

Figure 1. Subselective magnification angiograms of the rabbit ICA demonstrate (a) the circle of Willis and the MCA and ACA (arrow and arrowhead, respectively) and (b) occlusion of the MCA and ACA (arrow and arrowhead, respectively) after injection of three embolic spheres.
20% of the 30-min DDFPe group (n = 1 of 5), none of the 1-hour DDFPe group (n = 7), 20% of the 2-hour DDFPe group (n = 1 of 5), and none of the 3-hour DDFPe group (n = 6). The incidences of hemorrhage outside of stroke in these groups were 14%, 57%, 28%, 0%, 14%, 20%, and 17%, respectively.

The control rabbits at 7 hours had a numerically greater overall hemorrhage rate compared with 4-hour control animals, but not to a significant level (83% vs 29%; P = .10). The incidence of hemorrhage within stroke trended downward with treatment with DDFPe at 1 hour and every 90 minutes until euthanasia at 7 hours (P = .06) compared with control. Hemorrhage within stroke was seen in 67% of control animals (n = 4 of 6), none of the 1-hour DDFPe group (n = 6), and 60% of the 6-hour DDFPe group (n = 3 of 5). Hemorrhage outside of stroke occurred in these groups in 50%, 20%, and 33%, respectively, and did not differ between groups (P = .82).

Animals that received one DDFPe dose (n = 11), two doses (n = 25), three doses (n = 7), four doses (n = 8), and zero doses (ie, controls; n = 13) all survived to scheduled euthanasia without apparent adverse events.

DISCUSSION

The search for a neuroprotectant agent to use in acute stroke has been a high priority for many years. The parallel search for blood substitutes has included hemoglobin substitutes and PFCs in liquid form. Numerous studies of the use of these substances in hypoxia and ischemia have encountered side effects and severe complications, and all the agents studied have failed to translate into successful human therapy. Several oxygen free radical scavengers and other novel techniques have shown great promise in small animal stroke models, usually in mouse or rat. None has yet translated into therapy of human stroke (4). Here, we test a novel oxygen transport approach in an embolic stroke model without the possibility of thrombolysis. This rabbit model of stroke is similar to a model used in the successful development of tissue plasminogen activator (TPA) stroke therapy (16). Although this model is more expensive than rats and mice, its advantage in scale may be important, and it must be noted that other success has translated into human results. This included prediction of the failure of the antioxidant NXY-059 in the Stroke Acute Ischemic NXY-059 Trial (17,18).

Blood has a limited capacity to deliver oxygen, in large part requiring red blood cells to transit capillaries. With decreased blood flow or occlusion, this limitation becomes critical, causing infarction with nearly immediate cell death in some areas and ischemic damage without immediate cell death in others. This threatened area is the penumbra. In many strokes, an ischemic penumbra of potentially viable brain tissue might be saved if oxygen could be delivered there.

Previous therapies including liquid PFC-based oxygen carriers have largely failed to compensate for oxygen deficits. However, DDFPe as a gas at body temperature transports many times more oxygen per weight volume than liquid PFCs (11). The intravenous dose of DDFPe is less than 1% of that of other PFC-based agents. The nanosized droplets and bubbles pass — like TPA — through spaces smaller than red blood cells and transport oxygen to ischemic areas blocked from whole blood flow. Other PFC agents require larger doses and are retained within the body on a long-term basis. In human pharmacokinetic studies, intravenous DDFPe as a single smaller dose is well tolerated, and is rapidly cleared by exhalation without significant residual or side effects (12). In rats and pigs, larger doses act for as long as 2 hours (19).

When given intravenously, DDFPe may “pause the clock” on the treatment window for several hours, acting as a bridge to further acute stroke therapies, which might be delayed far beyond current therapeutic time windows. The present rabbit study shows clear benefit in decreased stroke volume compared with untreated controls, not only when DDFPe is given before occlusion or in the hyperacute time period (ie, from 0 to 30 min), but also with delays of 1–3 hours. Whereas prestroke administration could model preventive therapy in high-risk procedures and 0–30-minute therapy could model iatrogenic ischemic episodes, the latter...
groups model the usual stroke therapy, which is more delayed (20). The continued improved outcome at 3 hours in the present study is very promising in clinical terms, as the most common human therapy, intravenous TPA administration, begins to lose efficacy in this time frame, and endovascular recanalization, which can be performed as long as 6 hours after onset, is limited to major medical centers. This 3-hour improvement raises the possibility of DDFPe actually reversing nonlethal damage in addition to halting further damage. The 7-hour model shows that the damage has progressed too far for statistically significant therapeutic benefit with these small sample sizes at 6-hour administration. Importantly, this model shows that administration at 1 hour can be carried successfully to 7 hours with multiple doses, a point beyond most current thrombolysis protocols now in use. Prolonged success may also be possible. However, safety of multiple large doses is unproven in humans and problematic in dogs, in which rapid doses of DDFPe caused pulmonary hypertension and severe symptoms (21).

Measurements of ICH rates 4 hours after stroke were similar in all groups. The trend for increased rates of hemorrhage in control rabbits at 7 hours suggests that this time window of several hours after onset is important in the development of microscopic bleeding. Particularly encouraging is the absence of ICH in the 7-hour group treated with DDFPe from 1 hour (15,22). This raises the possibility of a protective aspect in this therapy, but needs to be confirmed with larger numbers of animal studies (23).

Figure 3. Infarct volume at 4 hours versus DDFPe treatment time. Categorization of treatment times to model various clinical scenarios—pretreatment, hyperacute, and acute therapy—demonstrates improved outcomes compared with control. Whether DDFPe is used as a pretreatment (30 min before embolization), a hyperacute treatment (0–30 min after embolization), or an acute treatment (1–3 h after embolization), stroke volumes are significantly reduced (*P ≤ .021, Dunnett-adjusted comparison of ranks).

Table 1. Influence of DDFPe Treatment Start Time on Infarct Volume at 4 Hours

<table>
<thead>
<tr>
<th>Treatment Start Time</th>
<th>No. of Pts.</th>
<th>Mean ± SE</th>
<th>Median</th>
<th>Unadjusted</th>
<th>Dunnett-adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>3.57 ± 1.41</td>
<td>3.20</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pretreat*</td>
<td>7</td>
<td>0.64 ± 0.37</td>
<td>0.30</td>
<td>.008</td>
<td>.04</td>
</tr>
<tr>
<td>Immediate</td>
<td>8</td>
<td>0.75 ± 0.35</td>
<td>0.40</td>
<td>.010</td>
<td>.05</td>
</tr>
<tr>
<td>30 min</td>
<td>5</td>
<td>0.70 ± 0.32</td>
<td>0.40</td>
<td>.083</td>
<td>.32</td>
</tr>
<tr>
<td>1 h</td>
<td>7</td>
<td>1.03 ± 0.59</td>
<td>0.30</td>
<td>.012</td>
<td>.06</td>
</tr>
<tr>
<td>2 h</td>
<td>5</td>
<td>0.72 ± 0.50</td>
<td>0.40</td>
<td>.028</td>
<td>.12</td>
</tr>
<tr>
<td>3 h</td>
<td>6</td>
<td>0.48 ± 0.28</td>
<td>0.25</td>
<td>.008</td>
<td>.04</td>
</tr>
</tbody>
</table>

Note.—DDFPe = dodecafluoropentane emulsion.
* DDFPe administration starting 30 min before embolization.
† P values compare each treatment time to untreated controls.

In addition to ischemic and hemorrhagic acute strokes, clinical applications might also include pretreatment of high-risk cardiac and carotid surgeries or neurovascular or cardiac interventions, providing a few hours of improved tissue oxygenation during iatrogenic ischemic episodes. Many strokes, cognitive deficits, or myocardial infarctions caused by transient clot, bubbles, or hypoxia might be completely avoided. Gaseous emboli and hypoperfusion episodes associated with surgery and vascular or cardiac interventions are transient phenomena and may require no additional therapy after DDFPe treatment. As human single-dose experience appears safe, this testing could quickly progress.

In addition to the need to fully investigate the time course of effectiveness of DDFPe, another limitation of the present study is the lack of therapeutic dosage testing. These studies used established dose levels for sonographic imaging, and optimization of therapeutic dose levels in rabbits and humans is required. Although considerable benefit was demonstrated at the chosen dosage and time points, further studies that compare other artificial oxygen carriers and fully characterize the treatment effects are needed. Moreover, the use of DDFPe must be examined in a thromboembolic stroke model as a combination treatment with intravenous TPA thrombolysis, intraarterial interventions, or sonothrombolysis with microbubbles and ultrasound (US). Here, safety and synergistic or additive effects will be appraised. If continued preclinical research overcomes these limitations, human feasibility testing in acute stroke can rapidly advance.
Further research will be required to optimize human dosage, timing, efficacy, and safety. This will be facilitated by the previous study of DDFPe as a US contrast agent in more than 2,000 patients and its approval as a US contrast agent by the European Agency for the Evaluation of Medicinal Products (now known as the European Medicines Agency) (24,25). The current single dose is smaller than that used as a human contrast agent, and dose optimization for therapeutic uses and safety testing of multiple doses have not yet been performed. Although reports of DDFPe as a contrast agent were very positive, development stopped for economic reasons, and DDFPe is not commercially available at this time.

Intravenous DDFPe protects brain tissue from ischemia, possibly by decreasing the degree of hypoxia. It decreases infarct volumes in stroke, and the effect can be sustained for several hours with repeated doses. Safety in humans has been demonstrated. Further animal studies and rapid development as a therapeutic oxygen delivery agent during times of stroke, blood loss, ischemia, and hypoxia, and in some preventive situations such as high-risk procedures, are warranted.

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