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Efficacy of Perfluorodecalin as an Oxygen Carrier for Mouse and Rat Testes Perfused *in Vitro* (42505)

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Abstract. Perfluorodecalin is a superior artificial oxygen carrier because of its high oxygen dissolving capacity, low toxicity, and short retention times within tissues. However, the instability of perfluorodecalin emulsions has hindered its application in blood substitutes. The present study addressed two questions. First, is perfluorodecalin deleterious to the endocrine function of testes? This question was examined by comparing testosterone secretion by testes perfused *in vitro* with medium incorporating either perfluorodecalin or erythrocytes as oxygen carriers. Second, can stable emulsions of perfluorodecalin be attained with the new surfactant Butronic U-1? This question was approached by determining the stability of perfluorodecalin emulsions containing either Butronic U-1 or Pluronic F-68, a proven ineffective surfactant. The experimental results support the efficacy of perfluorodecalin emulsions as oxygen carriers for mouse and rat testes perfused *in vitro.* Perfluorodecalin emulsions formed with Butronic U-1 were stable during the 4-hr perfusions but not during long-term storage.

Perfluorochemicals are biologically inert liquids that dissolve large volumes of oxygen. Of the perfluorochemicals evaluated as artificial oxygen carriers, perfluorodecalin (FDC) fulfills the essential criteria of low toxicity and short retention times within tissues (1–3). The effect of perfluorodecalin on endocrine organs has not been assessed directly. Furthermore, the application of perfluorodecalin as a component of artificial blood has been hindered because of the instability of emulsions that are required to render the hydrophobic perfluorodecalin miscible with plasma substitutes (2, 4). The reported studies focused on the effect of perfluorodecalin on testicular endocrine function and the stability of perfluorodecalin emulsions incorporating a new surfactant, Butronic Polyol U-1 (BU-1).

Our previous experiments established testes perfused *in vitro* as valid models for the evaluation of perfluorochemical emulsions (5). During these initial experiments, we discovered that Butronic U-1 had been patented as an emulsifier that produced stable perfluorodecalin emulsions (US Patent 4,395,393, July 26, 1983, I. R. Schmolka). In the present experiments, perfluorodecalin + Butronic U-1 emulsions were tested for their endocrine toxicity by determining their ability to replace erythrocytes as oxygen carriers for mouse and rat testes perfused *in vitro.* Additionally, the results were compared to studies of perfluorodecalin emulsified with Pluronic F-68 (F-68), a commonly used surfactant for perfluorochemical emulsions (6).

The experimental data provide evidence that perfluorodecalin emulsions are efficacious oxygen carriers for testes perfused *in vitro.* Testosterone secretion was supported equally well by perfusion medium containing either perfluorodecalin + F-68, perfluorodecalin + BU-1, or erythrocytes as oxygen carriers. The results do not support the ability of Butronic U-1 to form stable emulsions of perfluorodecalin.

Materials and Methods. *Animals.* Male Sprague–Dawley rats (402 ± 12 g) and male BALB/cANNHSD mice (24 ± 0.3 g) were purchased from Harlan–Sprague–Dawley (Indianapolis, IN) and caged in groups of four. Feed (Teklad 4%, Teklad Industries, Madison, WI) and water were available at all times. The animal room was maintained at 23 ± 1°C with 14 hr light/24 hr. Animals (2–4 months old) were sacrificed by cervical dislocation immediately before orchidectomy.

Surfactant selection. Physicochemical values were determined for 11 nonionic surfactants (data not shown) that were generously selected and provided by Dr. I. R. Schmolka (BASF Wyandotte, Wyandotte, MI). Physicochemical parameters were determined as

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follows: viscosity (LVT viscometer with UL adapter; Brookfield Engineering, Stoughton, MA); colloid osmotic pressure or oncotic pressure (Colloid osmometer, Model 4100; Wescor, Logan, UT); osmolality (vapor pressure osmometer, Model 5130 B; Wescor); optical density (measured as absorbance of undiluted solutions at a wavelength of 550 nm; Spectronic 21, Bausch & Lomb, Rochester, NY); pH (Microprocessor ionalyzer, Model 901; Orion Research, Cambridge, MA).

Perfusion media. Perfusion media containing either perfluorodecalin or erythrocytes (Table I) were prepared according to procedures described previously (5, 7, 8). The primary difference between perfusion media was the oxygen carrier. Briefly, perfluorodecalin medium was produced by sonicating perfluorodecalin with either F-68 or BU-1. When the optical density of the emulsions plateaued, the emulsion was centrifuged, treated with resin, filtered, dialyzed, and mixed with KRB, Hepes, and BSA. All perfusion media were used within 7 days after preparation.

Perfusion protocol. Mouse and rat testes were perfused *in vitro* according to procedures described and validated previously (5, 7, 9). In summary, testes were isolated from adnexa and perfused with medium via the capsular artery. One testis from each animal was perfused with RBC medium while contralateral testes were perfused with either FDC + F-68 or FDC + BU-1 medium. Testes perfused with RBC medium served as controls. Perfusions were performed at scrotal temperature (32.5°C) and at physiological flow rates (mouse, 2 ml/hr; rat, 20 ml/hr). Mouse and rat testes weighed 94 mg and 1.4 g ($\bar{X}$), respectively. Venous effluent was collected for 1 hr (mouse) or 0.5 hr (rat) from testes stimulated *in vitro* with luteinizing hormone (LH, 100 ng/ml; NIADDK-oLH-24) for 3 hr (mouse) or 3.5 hr (rat).

TABLE I. COMPONENTS OF THE PERFUSION MEDIA[a]

	Perfusion media		
	FDC + F-68	RBC	FDC + BU-1
O_2 carrier	FDC, 30%	RBC, 25%	FDC, 30%
Surfactant	F-68, 2.5%	None	BU-1, 2.5%
Buffer	KRB + Hepes	KRB	KRB + Hepes
Oncotic agent	BSA, 3%	BSA, 3%	BSA, 3%
Antibiotic	Penicillin	Penicillin	Penicillin

[a] All percentages are wt/vol. FDC, perfluorodecalin, $C_{10}F_{18}$ (mol wt 462.1, BP 141–142°C; PCR Research Chemicals, Gainesville, FL); RBC, washed bovine erythrocytes; F-68 (Pluronic Polyol F-68) and BU-1 (Butronic Polyol U-1) (gifts of Dr. IR Schmolka, BASF Wyandotte); KRB, Krebs–Ringer bicarbonate buffer (22); Hepes (25 m*M*; Sigma); BSA, bovine serum albumin, Fraction V(A-9647, Sigma); penicillin-G, sodium salt (1000 IU/ml, Sigma).

The following parameters were monitored during perfusions employing perfluorodecalin medium: pH, osmolality, oncotic pressure, and viscosity (see section titled "surfactant selection" for methods description). Additionally, PO_2 was measured with a specific electrode (Orion Research). Particle sizes of perfluorodecalin emulsions were estimated by optical density measurements of 1:20 dilutions of media (wavelength = 550 nm). The validity of measuring the qualitative stability of perfluorochemical emulsion by optical density determinations has been supported (10, 11) although newer methods (12) provide more information about particle size distribution.

Testosterone radioimmunoassay. Testosterone in the venous effluent was quantified by a double-antibody radioimmunoassay procedure. The assay has previously been validated for use with 10- to 100-μl aliquots of venous effluent collected from testes perfused *in vitro* with medium containing erythrocytes or perfluorochemicals (5, 7). Testosterone is the predominant steroid secreted by mouse (13) and rat (14) testes perfused *in vitro.*

Surfactant determination. Estimates of surfactant micelle size were obtained by determining the passage of surfactants through dialysis tubing with different molecular weight cutoff values. Surfactants were detected by first mixing 10 ml of sample, 30 ml of glass-distilled water, and 10 ml of Meyer's reagent (1.36 g mercuric chloride, 5 g potassium iodide, 100 ml glass-distilled water). After 5 min, the turbidity of the solution was spectrophotometrically determined (wavelength = 640 nm; Spectronic 21) and compared to the absorbance values of 10-ml standard solutions containing different concentrations of the surfactant (0.1, 0.2, 0.4, and 0.8%, wt/vol).

Statistical analysis. The significance of the difference between means was determined by Student's *t* test (15). All values are presented as the means ± SE.

Results. *Surfactant physicochemical properties.* Preliminary studies revealed that perfluorochemical emulsions incorporating the commonly used surfactant, Pluronic F-68, had supraphysiological measured oncotic pressures. This observation prompted us to investigate the physicochemical properties of 11 surfactants in order to identify a surfactant with a low measured oncotic pressure. The tested surfactants could be grouped into three categories: (1) butronic polyols = polyoxybutylene–polyoxyethylene block copolymers, (2) pluronic polyols=polyoxypropylene–polyoxyethylene block copolymers, and (3) tetronic polyols=polyoxypropylene–polyoxyethylene block copolymers added to ethylenediamine (BASF Wyandotte technical bulletins).

Butronic U-1, a butronic polyol, was selected as the surfactant for our perfluorochemical studies because of its low measured oncotic pressure and availability. Afterward, the reported ability of Butronic U-1 to form stable emulsions of perfluorodecalin was discovered. Pluronic F-68 was chosen for comparison because it is commonly used as a perfluorochemical emulsifier (6). Although the measured oncotic pressure for 2.5% (wt/vol) Butronic U-1 solutions was low (5.9 mm Hg), that of Pluronic F-68 (60.8 mm Hg) was higher than rat plasma oncotic pressure (19 mm Hg). However, testes perfused with perfluorochemical medium containing 2.5% F-68 as the sole oncotic agent exhibited dilated extravascular spaces ($n = 4$). These histological observations suggested that the measured oncotic pressure of Pluronic F-68 was not exerted within the *in vitro* perfused testis and that unphysiological extravasation of fluid from the blood vessels occurred.

We hypothesized that the difference in the measured oncotic pressures of Pluronic F-68 and Butronic U-1 solutions was due to micelle formation since the average molecular weights of both surfactants (5400-BU-1; 8350-F-68) were lower than the 30,000 mol wt cutoff of the colloid osmometer membrane. Pluronic and tetronic polyols have been reported to have low critical micelle concentrations (6). The linearity of measured oncotic pressures for Pluronic F-68 concentrations of 0.1% (1.2 mm Hg) to 10% (191 mm Hg) confirmed the low critical micelle concentrations of Pluronic F-68. The size of micelles in Butronic U-1 and Pluronic F-68 solutions was estimated by determining the amount of surfactant that passed through dialysis tubing containing 2.5%, wt/vol, solutions of BU-1 or F-68. The filled dialysis tubing was equilibrated in deionized water for 3–6 days. Dialysis tubing with MWCO values ranging from 6000 to 50,000 (Spectra/Por 1, 4, and 6; Spectrum Medical, Los Angeles, CA) blocked the passage of both F-68 and BU-1 since the surfactant concentration in the dialysate was 0 ($n = 6$). These experimental data suggested that both surfactants formed micelles that were larger than spherical proteins of 50,000 mol wt.

Testosterone secretion by in vitro perfused testes. The endocrine function of the testes was assessed by determining the testosterone secretion rate. Testosterone secretion was not significantly different for mouse testes (Fig. 1) or rat testes (Fig. 2) perfused with medium containing FDC + F-68, FDC + BU-1, or RBC as oxygen carriers.

Stability of perfluorodecalin emulsions. The stability of perfluorodecalin emulsions was studied at two levels: (i) as a component of the perfusion medium, and (ii) during long-term storage. Data in Table II demonstrate that perfusion media containing perfluorodecalin emulsified with either F-68 or BU-1 did not significantly change during the 4-hr

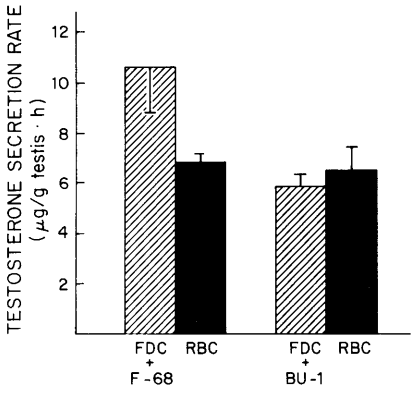

FIG. 1. Testosterone secretion by mouse testes perfused *in vitro* with three categories of media. Testes were perfused for 4 hr with medium containing erythrocytes (RBC) or perfluorodecalin (FDC) emulsified with either Pluronic F-68 (F-68) or Butronic U-1 (BU-1). All testes were maximally stimulated with luteinizing hormone. Results are reported as means ± SE for four testes.

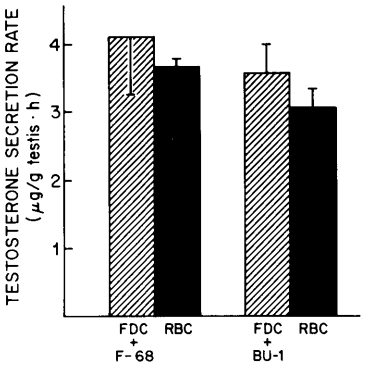

FIG. 2. Testosterone secretion by rat testes perfused *in vitro* with three categories of media. Testes were perfused for 4 hr with medium containing erythrocytes (RBC) or perfluorodecalin (FDC) emulsified with either Pluronic F-68 (F-68) or Butronic U-1 (BU-1). All testes were maximally stimulated with luteinizing hormone. Results are reported as means ± SE for four to six testes.

perfusion periods. However, FDC + F-68 and FDC + BU-1 emulsions either alone or incorporated in perfusion medium without BSA were increasingly unstable over a 25-day test period (Fig. 3). Experiments with test solutions stored at room temperature resulted in similar absorbance profiles; however, the rate of increase in absorbance was accelerated. Similar absorbance profiles were obtained for emulsions composed of 20% (wt/vol) FDC and 4.6% (wt/vol) F-68 or BU-1. Together, the experiments suggested that the FDC + BU-1 and FDC + F-68 emulsions were unstable due to coalescence under several conditions.

Discussion. The present studies demonstrate the efficacy of perfluorodecalin as an oxygen carrier for mouse and rat testes perfused *in vitro*. These experimental results represent the first direct evidence that perfluorodecalin is not deleterious to an endocrine organ. Perfusion media incorporating either perfluorodecalin or erythrocytes were equally effective in supporting testosterone biosynthesis and secretion. Testosterone secretion was selected for determination because of the series of oxygen-dependent enzymatic reactions required for testosterone biosynthesis (16). Additionally, the elevated secretion of testosterone by testes exposed to LH provided evidence that the Leydig cells retained the biochemical mechanisms required for response to LH stimulation.

The maintenance of testosterone secretion during the perfusion period suggests that perfluorodecalin medium provided the proper pH, osmolality, oxygen tension, viscosity, and oncotic pressure. Our previous reports accentuated the importance of these factors for successful testis perfusions (5, 8). However, the measured oncotic pressure of the two perfluorodecalin media varied significantly and was dependent solely upon the emulsifier. FDC + F-68 and FDC + BU-1 media had measured oncotic pressures of 34 ± 1 and 17 ± 0 mm Hg, respectively. Other investigators have observed the same phenomenon and have stated that Pluronic F-68 is unique because it exerts oncotic pressure (6, 17, 18). Other investigators reported that oncotic agents must be added to perfluorochemical emulsions containing Pluronic F-68 (20, 21). Our molecular size estimates for Pluronic F-68 and histological observations of testes perfused with F-68 as the sole oncotic agent support the latter findings and confirm the conclusions of Willcox and co-workers (19). They reported that Pluronic F-68 comigrates with albumin (mol wt 64,000) in acrylamide gel electrophoresis but that tritium-labeled Pluronic F-68 rapidly equilibrates between plasma and interstitial fluid. Together, these findings suggest that Pluronic F-68 and Butronic U-1 interact differently with the colloid osmometer membrane but simi-

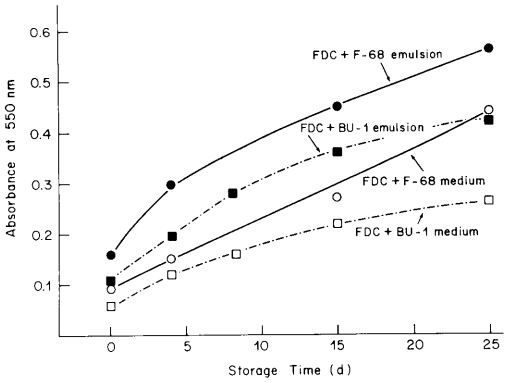

FIG. 3. Optical density measurements during 25-day test periods of 40% perfluorodecalin (FDC) emulsified with either 2.5% Pluronic F-68 (F-68) or 2.5% Butronic U-1 (BU-1). All % are wt/vol. Emulsions were tested alone or incorporated into perfusion medium without BSA. The test solutions were stored at 4°C and warmed to room temperature for the optical density measurements.

TABLE II. PHYSICOCHEMICAL PARAMETERS OF PERFUSION MEDIA BEFORE AND AFTER 4 hr PERFUSIONS OF MOUSE AND RAT TESTES[a]

	Perfusion media			
	FDC + F-68		FDC + BU-1	
	Before	After	Before	After
Viscosity, cP	2.2 ± 0.1	2.1 ± 0.1	2.7 ± 0.1	2.8 ± 0.1
Osmolality, mOsm	315 ± 8	315 ± 8	292 ± 29	292 ± 29
pH	7.4 ± 0.0	7.4 ± 0.1	7.4 ± 0.0	7.5 ± 0.0
Po_2 (arterial), mm Hg	—	456 ± 10	—	673 ± 57
Optical density, AU	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
n	3	3	4	4

[a] Values are means ± SE. FDC, perfluorodecalin; F-68, Pluronic F-68; BU-1, Butronic U-1.

larly with blood vessel walls. Neither appears to exert oncotic pressure within the organ.

Perfluorodecalin's eminence as an artificial oxygen carrier is accentuated by its inclusion as the predominant perfluorochemical in Fluosol-DA, the first perfluorochemical-based blood substitute tested in man (3, 20). However, the principal problem with perfluorodecalin is the instability of its emulsions. The stability of the Fluosol-DA emulsion is increased by the incorporation of a second perfluorochemical and freezing the emulsion during storage. Also, egg yolk phospholipid has been used as an effective emulsifier for perfluorodecalin (23; US Patent 4,423,077, December 27, 1983, H. A. Sloviter). Our attempts to obtain the purified egg yolk phospholipids from Vitrum AB, Stockholm, were not successful. Recent research has focused on the development of new surfactants that form suitable perfluorodecalin emulsions. Butronic U-1 is one surfactant that has been reported to form stable emulsions of perfluorodecalin. Our studies failed to demonstrate that FDC + BU-1 emulsions are stable. The emulsions in our study may have been prepared and stored differently from those reported in the patent by Schmolka since his methods were not fully described.

In conclusion, perfluorodecalin emulsions transport sufficient oxygen to maintain testosterone secretion by mouse and rat testes perfused *in vitro*. In addition, we have demonstrated the efficacy of emulsions consisting of perfluorodecalin and a new surfactant, Butronic U-1. Although we propose perfluorodecalin emulsions as excellent oxygen carriers for short-term organ perfusion *in vitro*, their *in vivo* application remains limited by emulsion instability.

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