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COMMUNICATIONS

Comparison of Sodium Polyanetholesulfonate with EDTA and Heparin Anticoagulants by Assessing Packed Cell Volume and Blood Smear Quality of Blood from Hybrid White Bass × Striped Bass

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Abstract.—Sodium polyanetholesulfonate, a synthetic polyanionic detergent compound commonly used as an anticoagulant in human blood culture transfer vials, was compared with EDTA and heparin, in two dilutions, in a pilot study of its anticoagulant abilities with blood from hybrid striped bass (white bass *Morone chrysops* × striped bass *M. saxatilis*). Packed cell volumes (PCVs) and blood smears made from the anticoagulant-treated blood samples were compared across time and with a control. Sodium polyanetholesulfonate kept the hybrid striped bass blood anticoagulated during the 24-h test period, but PCVs and smear quality were significantly different from the other treatments. Sodium polyanetholesulfonate does not appear to perform better than EDTA or heparin as an anticoagulatant for hybrid striped bass blood for hematology analysis.

Hematological evaluation of fish blood should be an integral part of routine diagnostic efforts to assess fish health. Hematologic analysis requires whole blood and usually involves the use of an anticoagulant. Mineral oil and wax and the chemical formulations of oxalates, citrates, EDTA (ethylenediaminetetraacetate), and heparin have been tried with varying success as anticoagulants for fish blood (Hesser 1960; Blaxhall 1972; Smit and Hattingh 1980). Although EDTA has been suggested as an acceptable piscine anticoagulant (Blaxhall 1972, 1973), some researchers have rejected its use because of cell lysis and swelling (Hattingh 1975; Smit et al. 1977; Smit and Hattingh 1980). Despite better preservation of cell morphology with EDTA, heparin is currently considered to be the anticoagulant of choice for fish in most work because of its anticoagulant ability and the low number of negative effects it has on the blood (Hesser 1960; Hattingh 1975; Smit and Hattingh 1980; Korcock et al. 1988).

Sodium polyanetholesulfonate (SPS), a synthetic polymer of anetholesulfonic acid (a polyanionic detergent), is a commonly used anticoagulant in human blood culture media (Merck & Co. 1989). It is unmodified by heating or dilute alkalis or acids, has good anticoagulant properties, and is anticomplementary and destructive to the bactericidal activity of human blood. Sodium polyanetholesulfonate interferes with leukocyte phagocytic activity and with the action of certain antibiotics by binding with them (von Haebler and Miles 1938; Stuart 1948; Traub and Lowrance 1970; Edberg and Edberg 1983). Sodium polyanetholesulfonate prevents blood from clotting by binding with cations, making them unavailable for use (Edberg and Edberg 1983). Currently, SPS is manufactured as a powder and routinely used for microbacterial studies. It is available as a microbiologic blood collection transfer vial (Vacutainer®, Baxter Diagnostics, McGraw Park, Illinois) that contains a 0.35% solution of SPS in 0.85% sodium chloride. A pilot study was conducted to evaluate sodium polyanetholesulfonate as a piscine anticoagulant and to compare it with EDTA and heparin anticoagulants by using packed cell volumes (PCVs) and blood smear quality.

Methods

Ten hybrid striped bass (white bass *Morone chrysops* × striped bass *M. saxatilis*; Pamlico Aquaculture Field Laboratory, North Carolina State University, Aurora), mean weight 336 g (SD, ±2.55), were used for this study. The fish were acclimated for 6 months to 1,500-L circular holding tanks. Water conditions at the time of the study were 18°C, pH 7.5, total hardness 975 mg/L, total alkalinity 1,230 mg/L, and dissolved oxygen 8.2–8.4 mg/L. The fish were fed a standard commercial diet (38% Hybrid Bass Grower 850161, Southern States Cooperative, Inc., Richmond, Virginia) at 2% body weight per day for 3 weeks prior to this study.

Blood samples were obtained from each of the fish for anticoagulant evaluation. Each fish was

netted from the holding tank and anesthetized by immersion in an experimental proprietary anesthesia solution currently under clinical trial. Once the animal lost its righting reflex and response to sensation (in approximately 3 min), it was placed in right lateral recumbency. Venipuncture was accomplished by a left lateral approach to the caudal vertebral vessel with a 3-mL syringe and 22-gauge needle. Blood (1–1.5 mL) was withdrawn within 30 s of needle insertion. Immediately upon completion of the venipuncture, the needle was removed from the syringe and 0.2 mL of blood was dispensed into each of four labeled containers containing either powdered disodium EDTA (Capiject®, Baxter Diagnostics; 1.55 mg Na_2 EDTA/mL blood), powdered lithium heparin (Microtainer®, Baxter Diagnostics; 25 IU lithium heparin/mL blood), 0.2 mL SPS solution (polypropylene microcentrifuge container producing a 1:1 dilution of SPS with blood), or 0.04 mL SPS solution (polypropylene microcentrifuge container producing a 1:5 dilution of SPS to blood). Sodium polyanetholesulfonate was used in the liquid form found in the blood collection vials (0.35% solution in 0.85% NaCl) and had an osmolality of 284 milliosmols per kg (mOsm/kg). Freshwater fish are known to have a serum osmolality around 295 mOsm/L (Stoskopf 1993).

As controls, two ammonium heparin capillary tubes and a blood smear were made directly from the syringe. After inverting the blood-filled containers 8–10 times, an untreated capillary tube was filled from each blood container, labeled 0 h and centrifuged at 13,000 × gravity (IEC MB Centrifuge, International Equipment Co. Needham Heights, Massachusetts) for 4 min. At 24 h after venipuncture, another untreated capillary tube was filled from each blood container in a similar manner and centrifuged. After capillary tubes were centrifuged, they were evaluated by using a PCV (%) card reader, and the plasma color was noted. Capillary tubes were used to evaluate PCV, plasma color, and evidence of red cell lysis and to look for changes in PCV.

At 1, 6, and 24 h after venipuncture, each blood container was mixed thoroughly, by inverting 8–10 times, and inspected for any gross signs of clotting. If a red mass (clot) failed to dissolve after mixing, the container was removed from the study. The inspection times of 1, 6, and 24 h were used to simulate ideal and realistic handling times (after sampling) when fish blood would be analyzed (with 1 h being ideal, 6 h allowing for transport, and 24 h allowing for overnight shipping). All samples were held at room temperature throughout the study. A blood smear was made with a drop extracted from each mixed unclotted container, air dried, and fixed in methanol. The smears were stained with a water-based Romanowsky stain (Leukostat™, Fisher Scientific, Orangeburg, New York). Ten to 20 fields from each stained smear were evaluated microscopically, under high dry (400 × magnification) and oil immersion (1,000 × magnification), for three categories of abnormality: white blood cell (WBC) clumps (three or more leukocytes together), disrupted cells (cells that were not intact or had lost clear cell or nuclear margins), and fibrin or protein strands. A smear was considered positive in a category if evidence of the abnormality was present. These smears were compared across the time periods and with the controls.

To facilitate statistical comparison, SPS packed cell volumes were corrected by multiplying the results with a factor to compensate for the SPS dilution with blood (SPS 1:1 dilution by a factor of 2; SPS 1:5 dilution by a factor of 1.2). The smear evaluation was a result of one person's subjective review. The control and anticoagulant 0-h and 24-h PCVs were evaluated for normality by using an F-test (JMP®, SAS Institute, Inc., Cary, North Carolina) before analysis. A paired Student's t-test for equal groups was used to evaluate differences between PCV at the 0-h and 24-h inspection times for each anticoagulant. A one-way analysis of variance using Dunnett's method was used to compare the 0-h anticoagulant PCV results to the control results. A chi-square test with Fisher's exact two-tailed test, when expected cell sizes were less than five data points, was used to assess significant differences in the amount of WBC clumps, disrupted cells, and fibrin or protein strands between the control slides and 1-h anticoagulant slides. A chi-square test with Pearson's correlation was used to assess for a statistical differences among the inspection times of 1, 6, and 24 h in each of the categories of WBC clumps, disrupted cells, and fibrin or protein strands for each anticoagulant. Since the capillary tubes and slides were made from the same container at different times, the values obtained for each inspection time are not truly independent of each other, which must be taken into consideration when assessing statistically significant differences (Glantz 1992). No investigation into sampling variance was conducted in this study. A P-value of less than 0.05 was considered significant in statistical testing.

TABLE 1.—Packed cell volume (%) summary statistics for hybrid striped bass ($N = 10$) blood samples after treatment with four anticoagulant preparations; SPS = sodium polyanetholesulfonate.

Statistic	Control	EDTA		Heparin		SPS 1:1		SPS 1:5	
		0 h	24 h	0 h	24 h	0 h	24 h	0 h	24 h
Mean (%)	38.5	36.9	38.9[a]	37.5	37.6	40.6	42.2	40.2	39.6
SD (%)	2.55	1.79	2.77	2.46	2.07	3.27	4.47	2.67	2.33
Range (%)	36–44	35–41	36–45	34–43	35–41	36–46	34–50	36–46	36–44

[a] Significant difference between 0 and 24 h, $P = 0.002$ (paired Student's t-test).

Results

The control PCV for the 10 hybrid bass ranged from 36.0% to 44.0% (mean 38.5%, SD ±2.55%. Table 1). A significant difference existed between the 0-h and 24-h times ($P = 0.002$) for EDTA. When the control PCVs and the anticoagulant PCVs at the 0-h inspection time were compared, no significant differences existed. Three of the heparin blood containers were clotted at the 24-h examination. The clots were small red masses of blood that did not dissolve upon shaking the containers and were removed by adherence to a wooden stick. These 24-h results were removed from the study. Three 0-h EDTA samples, one 24-h heparin sample, and five 24-h SPS 1:5 samples had a slightly pink-tinged plasma after centrifugation.

The number of blood smears positive for each category of abnormality (WBC clumps, disrupted cells, and fibrin or protein strands) from each of the 10 fish for the control, four anticoagulants, and each time period were recorded (Table 2). No significant differences occurred between the control smears and the 1-h inspection time smears for EDTA and heparin for any of the three abnormal blood smear categories. White blood cell clumps ($P < 0.01$) and the number of disrupted cells ($P < 0.01$) in SPS 1:1 dilution smears after 1-h were significantly different from those of the control smears. The number of disrupted cells in the SPS 1:5 dilution ($P < 0.01$) was significantly different from that of the control smears. When the smears were analyzed for significant differences among the three inspection times EDTA had no differences, heparin had a significant difference in the disrupted cell category ($P = 0.02$), and SPS 1:5 dilution had a significant difference in the fibrin or protein strands ($P = 0.01$) category. For SPS 1:1 dilution, each slide had WBC clumps and disrupted cells but very little fibrin or protein strands present. These responses did not change across inspection times thus were determined to have no statistically significant differences.

TABLE 2.—Number of blood smears (N = 10, except as noted) positive for different clotting characteristics after treatment with various anticoagulant preparations. Sodium polyanetholesulfonate (SPS) was diluted with blood at a 1:1 or 1:5 (SPS : blood) ratio.

Hours post-treatment	Clotting characteristic[a]	Control	Anticoagulant			
			EDTA	Heparin[b]	SPS 1:1	SPS 1:5
1	WBC clumps	0	1	4	10[c]	1
	Disrupted cells	4	8	8[d]	10[c]	10[c]
	Fibrin strands	1	5	3	1	2[d]
6	WBC clumps		0	1	10	4
	Disrupted cells		6	2[d]	10	7
	Fibrin strands		6	4	1	1[d]
24	WBC clumps		0	2	10	1
	Disrupted cells		5	2[d]	10	8
	Fibrin strands		9	2	1	7[d]

[a] WBC = white blood cell.

[b] $N = 7$ for the 24-h time period.

[c] Significantly different from control, $P < 0.01$ (chi-square, Fishers exact 2-tailed test).

[d] Significantly different over time periods, $P < 0.05$ (chi-square, Pearsons correlation).

Changes not included in the statistical analysis were noted with the anticoagulant blood smears. At 24 h, four of the EDTA smears, six heparin smears, and seven of the SPS 1:5 dilution smears contained pyknotic white cells. A single 1-h blood smear of SPS 1:5 dilution and one 24-h heparin sample had small thrombocyte clumps present. Overall stain quality did not appear to change among anticoagulants or inspection times.

Discussion

Fish red blood cells have been noted to swell and lyse, especially when using certain concentrations of various anticoagulants (Hattingh 1975; Smit and Hattingh 1980; Korcock et al. 1988). Pink plasma in several of the specimens examined in the present study were indicative of hemolysis that could lower the PCV (Schalm 1986a, 1986b), however, a significant difference in the PCV during the 24-h period was only noted with EDTA. No change in the PCV was noted during 24 h in SPS 1:5 despite indications of hemolysis. Swelling of the red cells possibly maintained the PCV level

across time, as has been noted in other anticoagulant studies (Hattingh 1975; Smit and Hattingh 1980; Korcock et al. 1988). This possibility could lead to a false representation of the stability of the red cell volume in the SPS 1:5 anticoagulant. The lack of hemolysis and a constant PCV for 24 h indicates a reliable PCV with the heparin and SPS 1:1 dilution samples. No significant change was noted between the control and the 0-h PCVs, possibly due, in part, to good (proper) handling of samples and to no immediate effect of the anticoagulant on the blood cells (Blaxhall 1973). The mean control PCV for the hybrid striped bass in this study was similar to the PCVs found in other hybrid striped bass (Hrubec et al. 1996).

The blood sample containers in this study were designed for human use and were held at room temperature throughout the study. It has been noted that fish blood clots more quickly at higher temperatures (Korcock et al. 1988). Three of the heparin samples clotted by the 24-h observation time, indicating that heparin is unsuitable for use in hybrid striped bass when refrigeration is not available for long-term storage of samples. Reports of other studies have not noted this effect in other fish species (Hattingh 1975; Smit and Hattingh 1980; Korcock et al. 1988).

Blood smear quality is important when including a white cell differential in the hematological analysis. Ideally, blood smears should be made from the syringe without anticoagulant and before clotting occurs (Stoskopf 1993). Anticoagulant with EDTA has been noted to have the best cell preservation and tinctorial properties in some fish blood (Blaxhall 1972) although heparin is also useful (Hesser 1960). In the present study, both EDTA and heparin produced slides of the same quality as the control method in all three clotting categories at the 1-h inspection time. Blood cells treated with EDTA were also preserved for substantially longer than 24 h, whereas heparin-treated samples had evidence of disrupted cells. The SPS-treated samples had an increased number of disrupted cells (1:1 and 1:5 dilutions) and WBC clumps (1:1 dilution) on the blood smears when compared with the control. Thus, SPS made the slide quality less than desirable at the 1:5 dilution and unacceptable at the 1:1 dilution.

Proper handling of samples is important for obtaining suitable samples for evaluation. The authors were able to draw the blood into a syringe without anticoagulant, at room temperature, and to process the samples before they clotted. The success of this procedure depended on the use of an anesthetic and an atraumatic venipuncture technique, and it emphasized the ability to work quickly before clotting cascade occurred (Klontz and Smith 1968). The lack of thrombocyte clumps present on the majority of the blood smears indicated that this variable was not a factor in these studies.

In summary, there still remains no ideal anticoagulant for use in all piscine species. Individual anticoagulant characteristics and piscine species red and white cell response to an anticoagulant must be kept in mind when an anticoagulant is chosen for use in a hematological study (Hattingh 1975; Smit et al. 1977). Sodium polyanetholesulfonate samples of both dilutions remained anticoagulated for a 24-h period, whereas several heparin samples clotted. However, SPS used at a dilution of 1:5 with blood had indications of hemolysis at 24 h and did not produce as good a quality of slides as did EDTA or heparin; SPS at the 1:1 dilution had a stable PCV for 24 h but very poor cell preservation and slide quality. Sodium polyanetholesulfonate has potential for use as a hybrid striped bass anticoagulant but does not appear to provide important advantages over EDTA or heparin for hematology studies.

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