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Source: Journal of Wildlife Diseases, 12(1) : 77-82
Published By: Wildlife Disease Association
URL: https://doi.org/10.7589/0090-3558-12.1.77

BLOOD CHANGES IN BROOK TROUT INDUCED BY INFECTION WITH *Aeromonas salmonicida*

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Abstract: Furunculosis was induced in brook trout, *Salvelinus fontinalis,* by experimental inoculation with *Aeromonas salmonicida.* Total protein, hemoglobin, sialic acid, fatty acids, triglycerides, cholesterol, inorganic-phosphorus, acid-soluble phosphorus, and lipid-phosphorus decreased in the blood of the infected fish while amino acids, urea, total creatinine, ammonia, and glucose increased. Pyruvic acid, lactic acid, and ascorbic acid values showed no significant change.

INTRODUCTION

Furunculosis is a highly fatal epizootic disease of salmonid fishes caused by *Aeromonas salmonicida,* a non-motile, gram-negative microorganism. Herman[8] reported that the biochemistry of *A. salmonicida* is largely unknown, but recent studies have been facilitated by the development of chemically defined media suitable for growth of this pathogen.[6,14] Since *A. salmonicida* requires arginine,[14] the role of this amino acid in nutrition,[17] its metabolic pathway,[18] the biosynthetic pathway of pyrimidines from arginine,[19] and the stimulatory effect of arginine on melanin pigment production[20] by *A. salmonicida* have been determined. The present experiments were designed to equate *in vitro* nutritional requirements of *A. salmonicida* with the biochemical changes occurring *in vivo* during an infection. Brook trout, *Salvelinus fontinalis,* were infected with *A. salmonicida* and the changes in blood composition determined to provide information relevant to diagnosis of this and similar diseases.

MATERIALS AND METHODS

Culture and Medium

The culture of *A. salmonicida* is a recent isolate from a brook trout at a southwestern Washington trout hatchery and was obtained from Mr. D. P. Anderson, Bureau of Sports Fisheries and Wildlife, Western Fish Disease Laboratory, Seattle, Washington, U.S.A. Cultures were maintained on trypticase soy agar medium.

Fish

About 400 brook trout, each weighing 100 g (av.), were used in this study. They were kept in circulating tanks measuring 1.83 m inside diameter and 1.22 m in height. Each tank would accommodate about 200 fish. Source of water for these tanks was a dechlorinated municipal supply. Water flowed continuously through the tanks and was maintained at 9 C. Fish were fed commercial trout pellets twice daily.

For each experiment 20 fish were removed from the holding tanks and placed, 10 each, into smaller circular tanks measuring 0.31 m inside diameter by 0.61 m in height. These tanks were supplied with aerated water at a temperature of 10 C over the experimental period.

Anaesthesia

Fish were anaesthetized with tricaine methane sulphonate (MS 222) at a concentration of 40 mg/l. Immobilization required about 10 min. They were then inoculated or bled as quickly as possible to reduce stress.

Inoculation

Bacterial suspensions for inoculation were made by washing cells from a 48 h culture in physiological saline. Cells were diluted to 0.05 optical density (O.D.) at 655 nm furnishing approximately 2.2 x 10^8 bacterial cells/ml. One-tenth ml of suspension was inoculated intramuscularly anterior to the dorsal fin. Control fish were injected with saline. Throughout the experimental period (3 days), fish were not fed.

Preparation of Blood

Samples for whole-blood or serum analysis were obtained by amputating the tail. Blood for whole blood analysis was collected in centrifuge tubes coated with dried heparin (0.2 mg/ml blood). Blood for serum analysis was collected in centrifuge tubes, allowed to clot at room temperature, chilled in ice, and centrifuged prior to removal of the sera.

Analytical Methods

Total protein was estimated using the Biuret reaction.[21] Urea, uric acid, total creatinine, amino acid-nitrogen, inorganic-phosphorus, acid-soluble phosphorus, lipid-phosphorus and total cholesterol were determined according to Hawk *et al.*[7] Hemoglobin, ammonia, ascorbic acid, total lipids, total fatty acids, free fatty acids, triglycerides, and free cholesterol were assayed according to Natelson.[18] Glucose was estimated by using orthotoluidine reagent[22] while pyruvic acid and lactic acid were assayed by a simple enzymatic procedure.[23] Sialic acid was determined by the alkali Ehrlich method.[1]

Data

The data were analyzed for statistical significance. The number of lots for control and infected fish was 4 and the samples in each lot was 10.

RESULTS AND DISCUSSION

Results are given in Table 1. Three days after inoculation total serum protein in infected brook trout was one-third lower than controls. Protein was 2.20 g/100 ml in uninfected trout and 1.50 g/100 ml in infected trout. Ulcerative Dermal Necrosis (UDN) also causes a reduction in total serum protein level in *Salmo trutta* L.[12] This may be a common response to disease because Yamashita[25] reported that the amount of serum protein in unhealthy Rockfish, *Sebasticus marmoratus,* was less than in normal specimens. Moreover, serum protein concentration in diseased salmon, *Salmo salar,* was less than the concentration in healthy salmon.[11]

TABLE 1. Biochemical comparison of blood from brook trout infected with **Aeromonas salmonicida** and from uninfected controls.

Component	Sample	Control		Infected		Level of significance
		X	Sm	X	Sm	
Total protein (g/100 ml)	Serum	2.2	0.4	1.5	0.3	S
Hemoglobin (g/100 ml)	Blood	9.11	1.8	6.02	1.2	S
Urea (mg/100 ml)	Blood	1.5	0.3	3.2	0.5	S
Uric acid (mg/100 ml)	Blood	1.0	0.1	1.2	0.1	S
Total creatinine (mg/100 ml)	Blood	1.6	0.2	2.2	0.3	S

Table 1 (Continued)

Component	Sample	Control		Infected		Level of significance
		X	Sm	X	Sm	
Amino acid-N (mg/100 ml)	Blood	19	2.1	28	3.5	S
Ammonia (mg/100 ml)	Serum	0.1	0.01	0.15	0.01	S
Glucose (mg/100 ml)	Blood	55.8	9.5	83.9	16	S
Pyruvic acid (mg/100 ml)	Blood	0.25	0.02	0.21	0.02	N.S.
Lactic acid (mg/100 ml)	Blood	5.37	0.6	6.61	0.8	N.S.
Ascorbic acid (mg/100 ml)	Blood	0.51	0.04	0.59	0.04	N.S.
Sialic acid (mg/100 ml)	Serum	96.5	19	52.5	11	S
Total lipids (mg/100 ml)	Serum	1104	220	768	150	S
Total fatty acids (mg/100 ml)	Serum	381	61	203	38	S
Free fatty acids (mg/100 ml)	Serum	63	7.1	37	4.5	S
Triglycerides (mg/100 ml)	Serum	290	55	230	35	S
Total cholesterol (mg/100 ml)	Serum	240	31	118	19	S
Free cholesterol (mg/100 ml)	Serum	80	11	32	6	S
Inorganic—P (mg/100 ml)	Serum	18.8	3.1	16.4	2.8	N.S.
Acid soluble—P (mg/100 ml)	Serum	23.2	4.1	20	3.5	S
Lipid—P (mg/100 ml)	Serum	19.2	3.7	12	1.6	S

X: mean

Sm: Standard deviation

S: Significant; N.S.: Not significant

Test t: Significant difference at P=0.05

Foda[4] reported that decreases in hemoglobin levels in hatchery-reared juvenile Atlantic salmon, *S. salar,* were associated with a severe *Aeromonas* infection.[15] In our experimentally infected brook trout, hemoglobin dropped from 9.11 g/100 ml to 6.02 g/100 ml. The reduction of hemoglobin may be attributable to hemorrhage or lysis of red blood cells following infection.

The non-protein nitrogen content of blood in infected fish increased. Amino acid nitrogen increased from 19 mg/100 ml to 28 mg/100 ml, while urea increased to 3.2 mg/100 ml, compared to 1.5 mg/100 ml in uninfected fish. Uric acid, total creatinine, and ammonia in infected fish were also higher than in uninfected fish. Similar results were observed by Field *et al.*[3] who reported that carp, *Cyprinus carpio,* infected with *A. salmonicida* showed marked changes in the non-protein nitrogen fraction of the blood. Yamashita,[25] investigating changes of serum urea nitrogen in Rockfish with ulcers, found that a higher value of urea nitrogen was obtained when the ulcer first developed and that a several-fold increase occurred after the fish was seriously affected. Glucose in infected trout was higher than in controls, while sialic acid was lower. Pyruvic acid, lactic acid and ascorbic acid showed no significant change. Field *et al.*[3] demonstrated that 3 days after inoculation blood sugar in four carp infected with *A. salmonicida* dropped from 100 mg/100 ml to a low of 5.8 to 12.3 mg/100 ml.. In contrast blood glucose levels in infected brook trout was always higher than controls. However, this difference may be the result of species response to the infection.

Serum sialic acid in infected fish was lower than in uninfected fish, suggesting the pathogen possibly interferes with glycoprotein. Tess and Kempf[24] observed a decrease in bound sialic acid from chicken embryos infected with influenza virus. Their observations suggest that viral enzyme combines with and cleaves glycoprotein on the cell surface before penetrating.

Profound alterations occurred in serum lipids of infected fish. Concentrations fell from 1104 mg/100 ml to 768 mg/100 ml. Total fatty acids, free fatty acids, triglycerides, total cholesterol and free cholesterol were also somewhat lower. The decrease in lipid fraction after infection could be a result of one or more factors: (1) decrease in the rate and amount of lipid synthesis; (2) increase in the clearance of lipids from serum; (3) less release from tissues and organs into the blood and (4) enzymatic action of *A. salmonicida* inducing a change in level. Alteration in serum lipids have been reported in other infections. Gallin *et al.*[5] reported that rabbits infected subcutaneously with *Escherichia* coli developed hyperlipemia related to increased serum levels of free fatty acids early in the infection and later as a result of increased levels of triglycerides. Rabbits challenged subcutaneously with *Staphylococcus aureus*[5] had normal levels of serum lipids during the acute febrile period but after 24 h developed hyperlipemia related to hypertriglyceridemia. Alteration of serum lipids in the rabbit following bacterial infections was studied by Farshtchi and Lewis.[2] Anthrax infections produced slight changes in the serum lipids; tularemia infections produced drastic changes in the serum lipids; and pneumococcal infections increased cholesterol 2.5 times, free fatty acids more than doubled, triglycerides increased 9.5 times, and lecithin increased almost 4-fold.

The phosphorus content of serum samples from infected fish decreased significantly. Lipid phosphorus level decreased from 19.2 mg/100 ml to 12 mg/100 ml, while acid-soluble phosphorus decreased to 20 mg/100 ml as compared to 23.2 mg/100 ml in uninfected fish. Kato and Watanabe[9] reported that a white wasting infectious disease leads to a remarkable change in phosphorus metabolism in the tissue of *Porphyra tenera;* the total phosphorus decreased since inorganic phosphorus values increased but the acid-soluble phosphorus and lipid-phosphorus decreased. Other factors can cause changes in phosphorus levels; for example, Phillips[16] found that changes in total phosphorus of whole brook trout were

corrected with diet and water temeprature. More phosphorus was utilized when the fish were on all meat diet than when fed a meat-meal diet. Colder water temperature favoured increased phosphorus levels. The effects of furunculosis on phosphorus metabolism in fish blood, however, are entirely different from those of malnutrition.

Field *et al.*[3] presented evidence for a severe hypoglycemia in fish with acute furunculosis. They suggested that the microorganism utilizes blood sugar and the hypoglycemic shock thus induced contributes to the mortality. This is not in agreement with findings in the present study. Several other possibilities also have been offered to explain the pathogenesis of *A. salmonicida*. Most workers feel that the pathogenicity is due to a prompt growth of the bacteria in tissues. This interferes with blood supply resulting in necrosis. Klontz *et al.*[10] reported that *A. salmonicida* produces an extracellular leucocytolytic substance that destroys the inflammatory reaction of fish. The data presented here suggests that the initial problem is proteolysis of muscle. The increase in amino acids and other nitrogen compounds indicates an extensive degeneration of muscle protein. Arginine can be utilized by the bacteria as a ready source of energy.[17,18] Other amino acids also may be used for growth *in vivo*. Later in the infection, a swollen necrotic area with intensive hemorrhage appears at the site of inoculation, suggesting that the bacteria may have invaded the circulatory system and thereby directly altering the components.

Acknowledgement

The authors thank Mr. D. P. Anderson for supplying the culture of *A. salmonicida*.

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Received for publication 6 November 1974