J. Parasitol., 87(1), 2001, p. 194–202
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THE IN VITRO EFFECTS OF ISOMETAMIDIUM CHLORIDE (SAMORIN) ON THE PISCINE HEMOFLAGELLATE *CRYPTOBIA SALMOSITICA* (KINETOPLASTIDA, BODONINA)

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ABSTRACT: Isometamidium chloride (Samorin) is therapeutic in rainbow trout (*Oncorhynchus mykiss*) during preclinical and chronic cryptobiosis. However, the toxic mechanism of isometamidium on *Cryptobia salmositica* has not been elucidated. The objective of the present study was to examine the in vitro effects of isometamidium on *C. salmositica.* Under in vitro conditions, isometamidium chloride reduced the infectivity of *C. salmositica* suspended in whole fish blood. It accumulated rapidly in the kinetoplast (within 1 min) and caused disruption and decantenation of kinetoplast DNA. The in vitro cryptobiacidal activity of isometamidium was reduced when parasites were incubated in medium containing serum supplement, suggesting that isometamidium also binds to plasma proteins. Isometamidium altered glycoprotein receptors (epitopes) for antibodies on the surface of *C. salmositica* and thus protected some of the parasites from lysis by complement-fixing antibodies. In vitro oxygen consumption and carbon dioxide production decreased in drug-exposed *C. salmositica,* with increased products of glycolysis, i.e., lactate and pyruvate, after exposure to isometamidium. This suggests that some *C. salmositica* switched from aerobic respiration to glycolysis when the mitochondrion was damaged by isometamidium.

Cryptobia salmositica (Kinetoplastida, Bodonina) causes disease and mortality in all *Oncorhynchus* spp. (see Woo, 1992, 1994), and transmission normally occurs through the bite of the blood-sucking leech *Piscicola salmositica* (Becker and Katz, 1965). The parasite causes significant mortalities in naturally infected postspawning rainbow trout (*Oncorhynchus mykiss*) and prespawning chinook salmon (*Oncorhynchus tshawytscha*) (Woo and Poynton, 1995). *Cryptobia salmositica* is considered a lethal pathogen in both seminatural and intensive salmon culture facilities on the west coast of North America (Bower and Margolis, 1984). Ardelli and Woo (1999) demonstrated that 1.0 mg/kg of isometamidium chloride (Samorin) delivered intramuscularly (i.m.) was therapeutic in experimentally infected rainbow trout (*O. mykiss*). The mechanism of isometamidium chloride toxicity was not elucidated.

Isometamidium chloride (isometamidium) is an amphiphilic cationic phenanthridinium and is highly positively charged under physiological conditions (Wang, 1995). The primary actions of phenanthridiniums are to block nucleic acid synthesis by intercalation between DNA base pairs (Wagner, 1971), leading to inhibition of RNA (Richardson, 1973) and DNA polymerases (Marcus et al., 1982), and thus inhibition of incorporation of nucleic acid precursors into DNA and RNA (Lantz and Van Dyke, 1972). Isometamidium is thought to exert its cytotoxic effects on trypanosomes by modulating glycoprotein biosynthesis (Casero et al., 1982) and lipid (Dixon et al., 1971) and ATP (Frank-Henderson et al., 1977) metabolism. The kinetoplast, but not nuclear DNA, was disrupted when trypanosomes were exposed to isometamidium (Newton, 1974). In addition, isometamidium can produce dyskinetoplastic trypanosomes; these are cells that retain the mitochondrial membranes but do not contain detectable kinetoplast DNA (Shapiro, 1993). Although it has been used as a trypanocidal drug for over 30 yr, the exact toxic mechanism of isometamidium has not been elucidated, although it is presumed to act in ways similar to other phenanthridiniums (Kinabo and Bogan, 1988).

The objectives of the present study were to examine the in vitro effects of isometamidium on the ultrastructure, biology, and infectivity of *C. salmositica.*

MATERIALS AND METHODS

In vitro culture of *C. salmositica*

A cloned strain of pathogenic *C. salmositica* was used to infect rainbow trout (*O. mykiss*) held at 11 C. The strain was initially isolated from the leech *P. salmositica* and cloned as described by Woo (1979). Blood from an infected trout was aseptically inoculated into sterile tissue culture bottles containing minimum essential medium (MEM) supplemented with Hank's salts, L-glutamine, 25 mM HEPES buffer, and 25% heat-inactivated fetal bovine serum (FBS) (Li and Woo, 1991). After 3 days (to allow red cells to settle), MEM containing *C. salmositica* was withdrawn from the flask and inoculated into sterile flasks containing MEM. Cultures were maintained at 11 C for no more than 8 wk as the parasite maintains its pathogenicity to trout in short-term culture (Woo and Thomas, 1991).

Localization of isometamidium in *C. salmositica*

Phase-contrast and fluorescent microscopy: C. salmositica (harvested from culture) were washed 3 times by centrifugation (15 min at 10,700 *g*) in cold-blooded vertebrate Ringer's solution (CBVR) and quantified using a hemacytometer (Archer, 1965). Parasites (1.0×10^6) were incubated in 1×10^{-4} mg/ml of isometamidium chloride for 1 min and then viewed using an Olympus BX60 microscope. The concentrations of isometamidium used in the present study were based on previous experimental work (Ardelli and Woo, 1999, 2000). Parasite images were obtained using a combination of phase-contrast (objective ×100) and fluorescent microscopy (objective ×100, excitation filter BP460-4090, dichromatic beam splitter DM500, and barium filter BA515IF). Fluorescent and phase-contrast images were compiled using Image-Pro Plus The Proven Solution and merged using Confocal Assistant 4.0 Image Software (Bio-Rad Laboratories, Mississauga, Ontario).

Electron microscopy: Parasites (1.0×10^6) were incubated in isometamidium (5×10^{-6} μg/ml, 5×10^{-5} μg/ml, and 5×10^{-4} μg/ml) and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate at 5-min intervals for 60 min. Samples were serially dehydrated to 70% ethanol and then treated with 0.5% *p*-phenylenediamine (Ledingham and Simpson, 1972) in 70% ethanol. Dehydration was continued to 100% ethanol and samples were embedded in Spurr's resin and polymerized at 60 C for 24 hr.

Samples were cut from resin, attached to stubs, and sectioned. Ultrathin sections corresponding to a gold interference color were cut using a Reichert microtome equipped with a diamond knife. Ribbons were collected from the water surface on cleaned, uncoated copper mesh grids and allowed to dry for 60 min. Sections attached to grids were stained using saturated uranyl acetate (7.7%) and acetone (1:1) for 5

Received 6 March 2000; revised 21 July 2000; accepted 21 July 2000.
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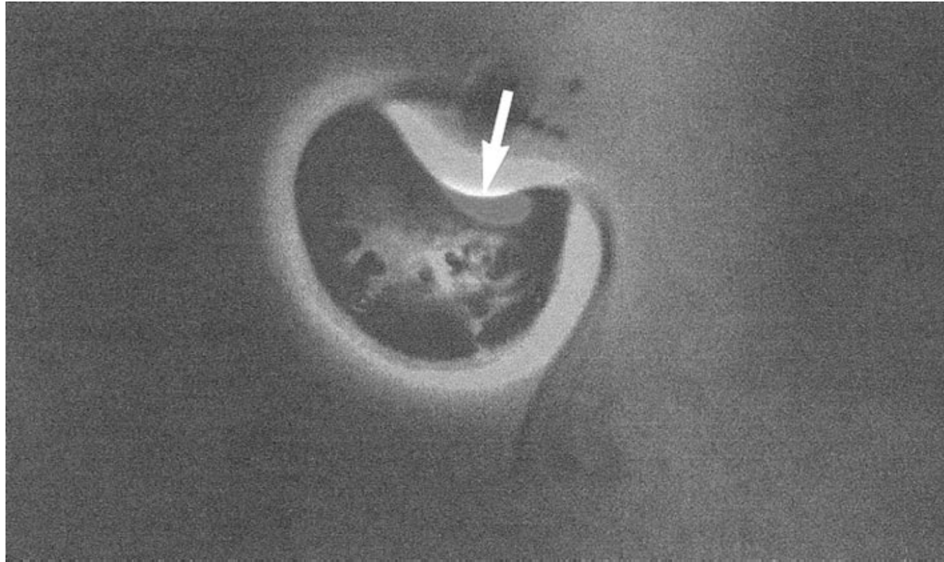


FIGURE 1. Phase-contrast and fluorescent microscopy showing accumulation of isometamidium in the kinetoplast of *Cryptobia salmositica.*

min and lead citrate for 1 min. Sections were examined using a JEOL 100CX electron microscope operating at 80 kV.

In vitro effects of prolonged isometamidium exposure on *C. salmositica* surface epitopes

Cryptobia salmositica was isolated from the blood of a rainbow trout and maintained in MEM (see above). The isolate was divided into 2 groups (group A and group B; n = 10 replicates/group). Group A was maintained in MEM without isometamidium and group B was maintained in MEM containing 1×10^{-5} μg/ml of isometamidium. Parasites were subcultured every 6 wk for a period of 8 mo. These isolates were used in an in vitro experiment to determine the effects of isometamidium parasite epitope(s) that activates complement-fixing antibodies.

Immune plasma was from 10 rainbow trout that had been vaccinated with *C. salmositica* and heat inactivated for 30 min at 40 C (Sakai, 1981). Heat-inactivated plasma (25 μl) was dispensed into a well (n = 10) in a cold microtiter plate. Twenty-five microliters of fresh rainbow trout complement and 25 μl of CBVR containing 500 living, washed *C. salmositica* from group A (cultured without isometamidium) or from group B (cultured with isometamidium) was added to the wells. The mixture was incubated at 11 C for 3 hr, and the microtiter plate was examined under a microscope (ocular ×10; objective ×10) for living or agglutinated parasites. Controls were parasites incubated in MEM, CBVR, immune plasma, and no complement, and complement and no immune plasma.

Effects of isometamidium on oxygen consumption

Nine tissue culture flasks were divided into 3 groups (groups C, D, and E; n = 3/group) and filled with 25.0 ml of MEM. Groups C and D were each inoculated with 100,000 *C. salmositica*; group E did not contain parasites. After 5 wk, flasks in group D were each inoculated with 2×10^{-5} μg/ml of isometamidium. Flasks were sampled weekly (0.2 ml) and the number of parasites, oxygen consumed, and carbon dioxide produced were measured. Oxygen consumption and carbon dioxide production were measured using an ABL500WO radiometer (London Scientific Ltd., London, Ontario). The ABL500WO radiometer is a fully automated, thermostated unit that is equipped with pH, pCO_2, pO_2, and reference electrodes. Group E (without parasites) was used to determine background levels.

Infectivity test for assessing in vitro sensitivity of *C. salmositica* in blood to isometamidium

Blood was withdrawn from the caudal vein of an infected rainbow trout and diluted (with uninfected whole trout blood) such that 25 μl of blood contained approximately 5,000 *C. salmositica.* The diluted blood was added to 40 wells of a 96-well cold microtiter plate. Isometamidium in phosphate-buffered saline (group G — 5×10^{-6} μg/ml, n = 10; group H — 5×10^{-5} μg/ml, n = 10; group I — 5×10^{-4} μg/ml, n = 10) was added to the blood. Controls (group F) were whole blood with *C. salmositica* incubated without isometamidium. Experimental and control groups were incubated for 3 hr at 11 C and then examined under an inverted microscope (ocular × 10 and objective ×10) for living parasites.

After incubation, the contents in each well in the microtiter plate (with isometamidium and *C. salmositica*) was withdrawn into a syringe, the well rinsed with 0.1 ml of Alsever's solution, and the contents inoculated intraperitoneally into a juvenile rainbow trout. Fish were bled (0.1 ml/fish) at 3 wk postinoculation (p.i.), and parasitemias were determined using a hemacytometer for high parasitemias (Archer, 1965) or the hematocrit centrifuge technique for low parasitemias (Woo, 1969).

In vitro effects of serum protein on the cryptobiacidal action of isometamidium

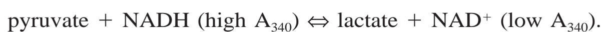
Cryptobia salmositica from cultures were washed 3 times in CBVR and diluted such that 25 μl of CBVR contained approximately 1,000, 10,000, or 100,000 parasites. Parasites were added to each of 10 wells in a microtiter plate and were exposed to the following treatments (n = 10/treatment): MEM (without serum supplement) containing 5×10^{-5} μg/ml of isometamidium, CBVR containing 5×10^{-5} μg/ml of isometamidium, rainbow trout plasma containing 5×10^{-5} μg/ml of isometamidium, deproteinized rainbow trout plasma, and whole blood containing 5×10^{-5} μg/ml of isometamidium. Controls consisted of MEM (with serum supplement), rainbow trout plasma, whole blood, and CBVR without isometamidium. The plates were incubated at 11 C and examined for living parasites using an inverted microscope (ocular ×10 and objective ×10).

Effects of isometamidium on protection of glycolytic end-products

Thirty tissue culture flasks were divided into 3 groups (J, K, and L; n = 10/group). Each flask contained 30.0 ml of MEM supplemented with 25% FBS and 5.0 mg/ml of glucose. To each flask in groups K and L was added 100,000 pathogenic *C. salmositica.* Group J had no parasites (controls). Group K was inoculated with 2×10^{-5} μg/ml of isometamidium at 5 wk after the start of the experiment. The number of parasites, glucose consumed, and pyruvate and lactate produced were determined for each culture. Controls (group J) were used to determine background levels attributed to the serum supplement.

FIGURE 2. Lesions in *Cryptobia salmositica* after in vitro exposure to isometamidium chloride. **a.** Kinetoplast of *C. salmositica* not exposed to isometamidium chloride. **b.** Condensation of kinetoplast DNA after exposure to isometamidium. **c.** Vacuole formation after exposure to isometamidium. **d.** Swelling of cristae after exposure to isometamidium. **e.** Vacuole formation in the cytoplasm after exposure to isometamidium. V, vacuole; C, cristae; K, kinetoplast.

Pyruvate produced was determined enzymatically and utilized lactate dehydrogenase to catalyze the following reversible reaction:



$$\text{pyruvate} + \text{NADH (high } A_{340}) \Leftrightarrow \text{lactate} + \text{NAD}^{+} \text{ (low } A_{340}).$$

In the presence of excess NADH substantially all pyruvate is converted to lactate. The reduction of absorbence at 340 nm due to oxidation of NADH to NAD^+ becomes a measure of the amount of pyruvate originally present in solution. An aliquot of 2.0 ml of MEM with parasites from flasks in each group (H, I, or J) was pipetted into a centrifuge tube containing 4.0 ml of cold 8% perchloric acid. The mixture was then placed at 4 C for 5 min to precipitate proteins. Proteins were pelleted (3 min at 10,600 *g*) and 2.0 ml of supernatant was placed in a cuvette with 0.5 ml of 1.5 M Tris buffer. After mixing, 0.5 ml (1.0 mg/ml in Tris buffer) of NADH was added and the absorbence was read at 340

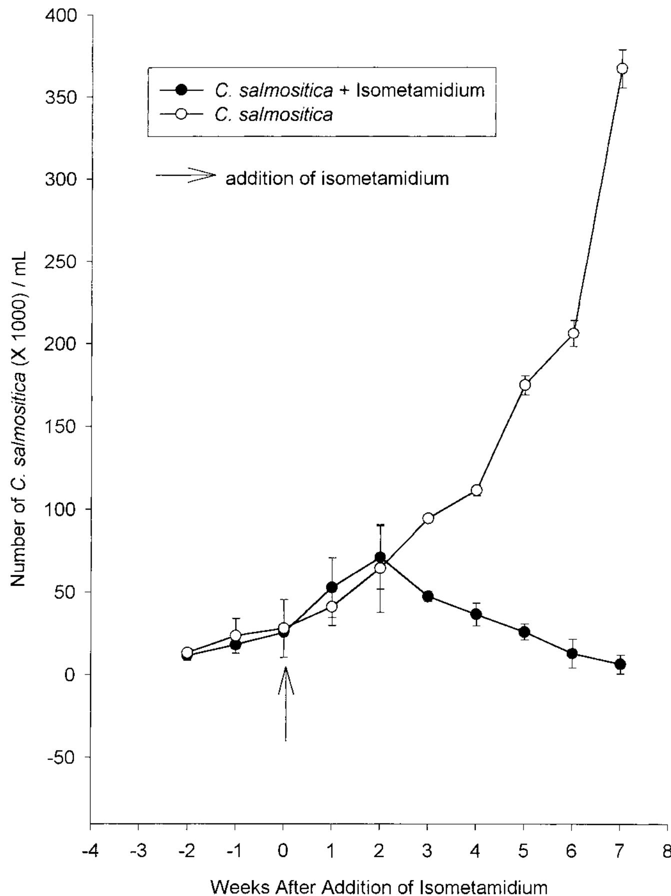


FIGURE 3. Multiplication of *Cryptobia salmositica* after in vitro exposure to isometamidium.

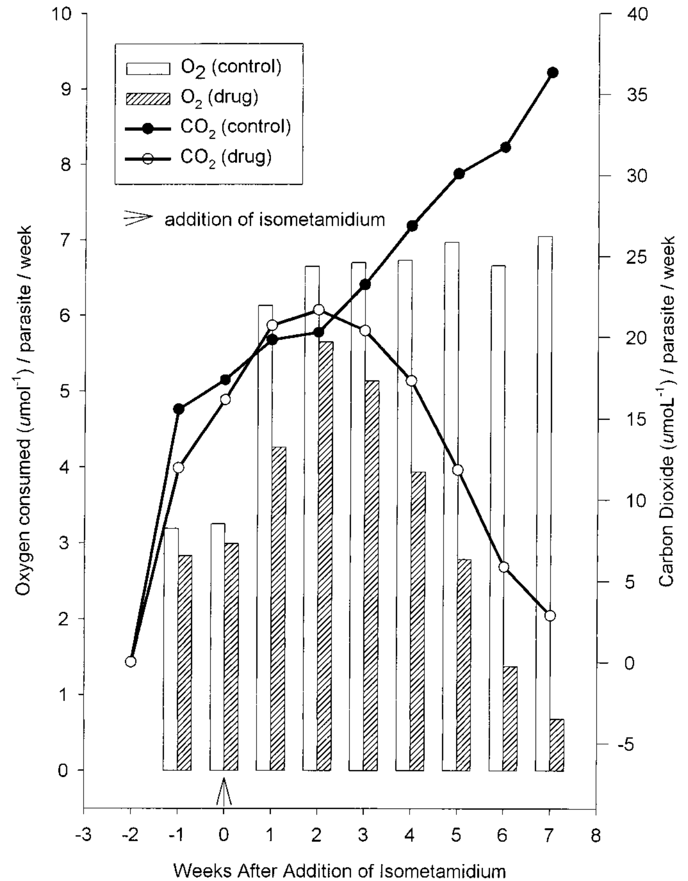


FIGURE 4. Oxygen consumption and carbon dioxide production in *Cryptobia salmositica* after in vitro exposure to isometamidium.

nm. Lactate dehydrogenase (0.5 ml of a 1,000-U/ml solution) was added, and after 5 min the absorbence was read at 340 nm and then reread at 5-min intervals until the decrease in absorbence was less than 0.001/min. Pyruvate (mg/dl) was determined according to the manufacturers' instructions (Sigma, St. Louis, Missouri).

Lactate was also determined enzymatically. The procedure was based on the principle that lactic acid is converted to pyruvate and hydrogen peroxide by lactate oxidase. In the presence of the hydrogen peroxide formed, peroxidase catalyzes the oxidative condensation of chromogen precursors to produce a colored dye with an absorption maximum at 540 nm. The increase in absorbence at 540 nm is directly proportional to the lactate concentration in the sample. A 1.0-ml aliquot of lactate reagent (containing 400 U/L of lactate oxidase, 2,400 U/L horseradish peroxidase, and chromogen precursors in 0.2 M glycine buffer; Sigma) was pipetted into a cuvette. A 10-μl volume of MEM from each flask (groups H, I, or J) was added to the cuvette and allowed to stand for 10 min. The absorbence was read at 540 nm against a blank containing no sample. The lactate (mg/dl) concentration in the sample was determined according to the manufacturer's instructions (Sigma).

Glucose was determined using a Sigma diagnostics test kit. The procedure is based upon the following coupled enzymatic reactions:

$$\text{glucose} + H_2O + O_2 \rightarrow \text{(glucose oxidase) gluconic acid} + H_2O_2$$

$$H_2O_2 + o\text{-dianisidine} \rightarrow \text{(peroxidase) oxidized } o\text{-dianisidine (brown).}$$

The intensity of the brown color measured at 425–475 nm is proportional to the original glucose concentration. Sample (0.5 ml of a 20-fold dilution) was added to a test tube containing a mixture of glucose oxidase, peroxidase, and *o*-dianisidine. The reaction was allowed to proceed for 30 min at 37 C, and the absorbance was read at 475 nm against a blank and a standard.

Statistical analysis

A 1-way analysis of variance (ANOVA) was used for data analysis. When the distribution was not normal and the variances unequal, an ANOVA on ranks was used. If the results were significantly different, a pairwise comparison was performed using a Student–Newman–Keuls test (for parametric data) or a Dunn's test (for nonparametric data). Significance was evaluated at $P \leq 0.05$.

RESULTS

Localization of isometamidium in *C. salmositica*

The kinetoplast of *C. salmositica* is the principal target of isometamidium. Fluorescence combined with phase-contrast microscopy demonstrated the initial and rapid (within 1 min of exposure) accumulation of the drug within the kinetoplast (Fig. 1).

The parasite not exposed to isometamidium had a large kinetoplast–mitochondria complex that contained kinetoplast DNA. The single mitochondrion was bounded by a double membrane, and it had many cristae (Fig. 2a). Ultrastructural modifications of the kinetoplast were observed after incubation in isometamidium. The first morphological change was a rapid disruption of kinetoplast but not nuclear DNA. The kinetoplast DNA appeared as a large, dense mass after a 5-min incubation in 5×10^{-6} μg/ml of isometamidium (Fig. 2b). At times, the cristae in the mitochondrion appeared swollen and distorted (Fig. 2c). Vacuoles appeared after 60 min, and the cytoplasm

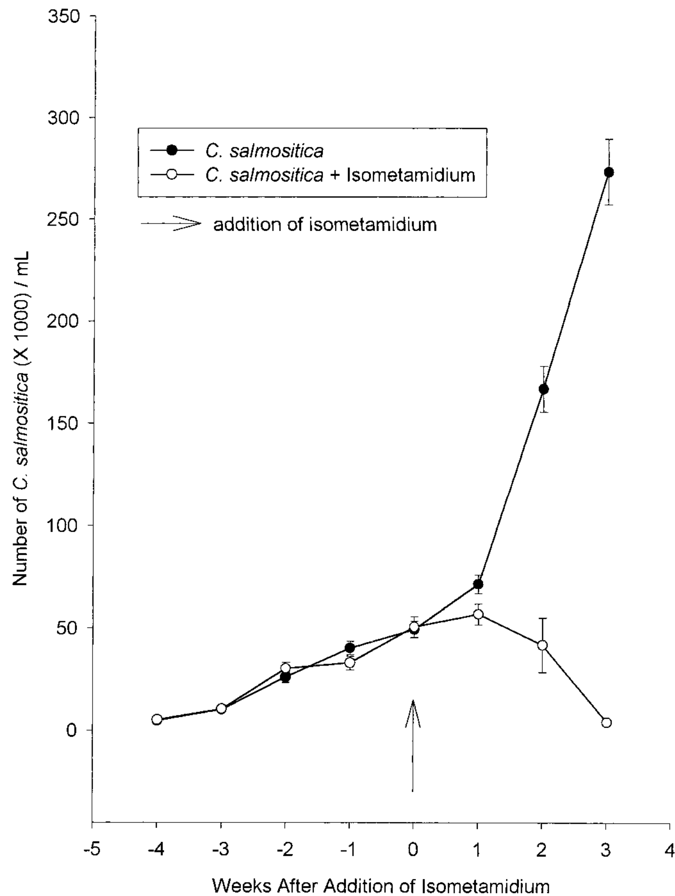


FIGURE 5. Multiplication of *Cryptobia salmositica* after in vitro exposure to isometamidium at 5 wk p.i.

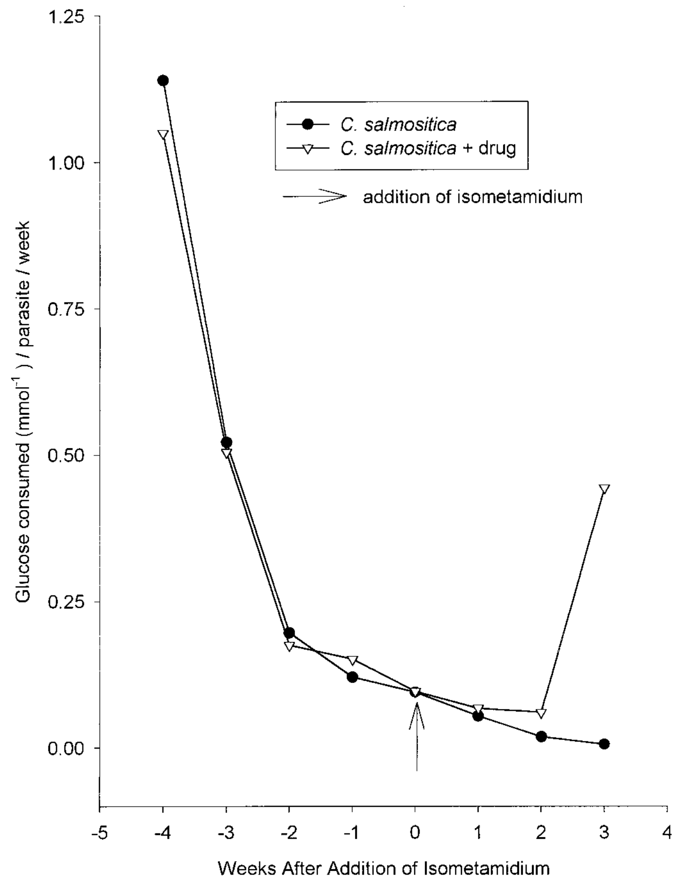


FIGURE 6. Glucose consumption in *Cryptobia salmositica* after in vitro exposure to isometamidium.

contained large, dark masses of debris (Fig. 2d–e). The severity of the changes seemed concentration dependent and were more obvious at 5×10^{-4} μg/ml of isometamidium.

Effects of isometamidium on surface epitopes of *C. salmositica*

Cryptobia salmositica maintained in MEM without isometamidium were lysed after incubation in heat-inactivated immune plasma and complement. Also, parasites were agglutinated by the immune plasma. In contrast, *C. salmositica* maintained in MEM (for 8 mo) containing isometamidium were not lysed by immune plasma and complement and were also not agglutinated by the immune plasma.

Effects of isometamidium on oxygen consumption

Control cultures (group C—without isometamidium) multiplied readily in MEM. Parasites in group D (exposed to isometamidium at 5 wk p.i.) multiplied readily before treatment and for 2 wk after drug exposure, after which it declined. Significant differences in parasite numbers were only detected between control cultures and cultures exposed to isometamidium 3–7 wk after addition of the drug (Fig. 3).

Oxygen consumption and carbon dioxide production did not differ significantly between drug-treated and non-drug-treated cultures prior to treatment. After treatment, oxygen consumption decreased in cultures treated with isometamidium (group D) but continued to increase, with increasing numbers of parasites, in untreated cultures (group C). The amount of oxygen consumed differed significantly between groups D and C at 4–7 wk after drug exposure (Fig. 4).

Effects of isometamidium on parasite metabolism

The number of parasites in all cultures (between 1 to 5 wk) prior to addition of isometamidium were not significantly different. At 1–3 wk after addition of the drug, the numbers of parasite in cultures with isometamidium (group K) were significantly lower than those cultures without the drug. Parasite numbers declined after drug exposure, whereas numbers continued to increase in cultures without the drug (Fig. 5).

Glucose consumption did not differ significantly between cultures (treated or nontreated) at 1–6 wk. At 2 wk after addition of isometamidium, drug-exposed parasites consumed more glucose, and this was significantly higher than glucose consumed by parasites in nontreated cultures at 3 wk after treatment (Fig. 6).

The amount of pyruvate produced by cultures did not differ significantly prior (1–6 wk) to drug exposure. At 2 and 3 wk after addition of isometamidium, pyruvate produced by parasites in drug-treated cultures were significantly higher than those in nontreated control cultures (Fig. 7).

Similarly, lactate produced did not differ significantly be-

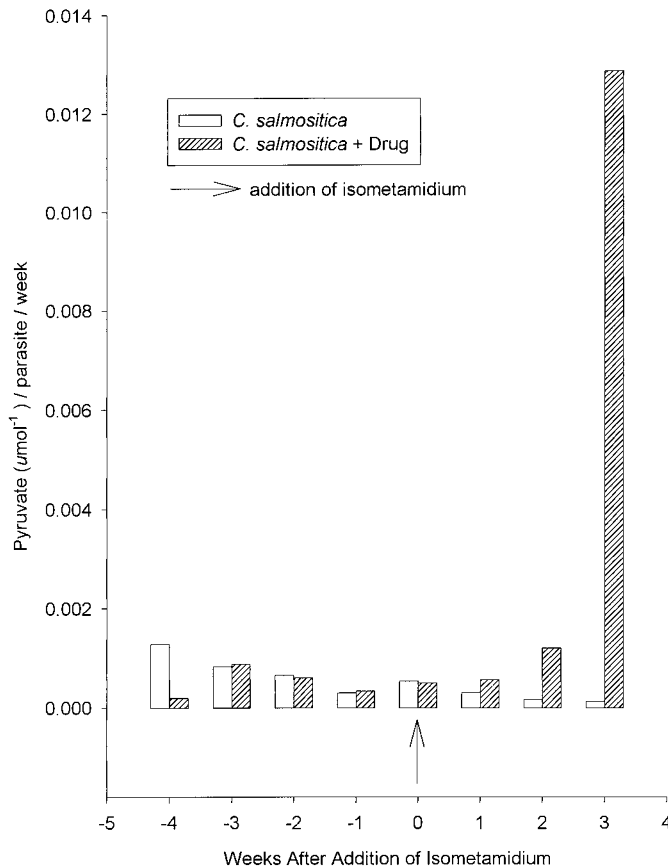


FIGURE 7. Pyruvate produced by *Cryptobia salmositica* after in vitro exposure to isometamidium.

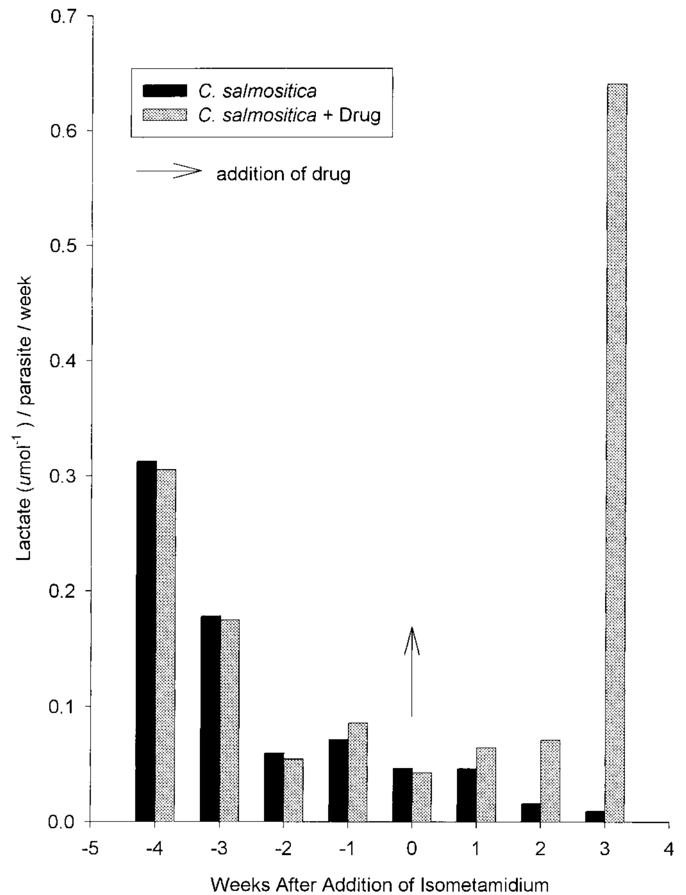


FIGURE 8. Lactate produced by *Cryptobia salmositica* after in vitro exposure to isometamidium.

tween cultures before treatment with isometamidium. After addition of the drug, lactate production increased in parasites in drug-treated cultures and was significantly higher than parasites in nontreated cultures at 2 and 3 wk after treatment (Fig. 8).

Infectivity of *C. salmositica* after in vitro exposure to isometamidium

The infectivity of *C. salmositica* was significantly reduced after in vitro exposure to isometamidium. As the concentration of isometamidium increased, the parasitemias and number of fish with a detectable infection decreased. At a concentration of 5×10^{-4} μg/ml only 50% of fish had a detectable infection, whereas 100% of controls were infected with *C. salmositica* (Table I).

In vitro effects of serum protein on the cryptobiacidal activity of isometamidium

The in vitro cryptobiacidal activity of isometamidium was reduced when parasites were incubated in medium containing plasma (either MEM with FBS, with trout plasma or with whole blood). Parasites incubated in medium without plasma supplement (MEM without FBS and deproteinized trout plasma) were lysed within 3 hr of incubation. In addition, as the number of *C. salmositica* (in medium with plasma supplement) increased, the cryptobiacidal effects decreased (Table II).

DISCUSSION

The present study demonstrated that isometamidium accumulated in the kinetoplast of *C. salmositica* and caused a rapid disruption of kinetoplast DNA. This is not unusual, as a number of anti-trypanosomal agents are known to bind DNA. These include ethidium bromide, a DNA intercalating agent, and the diamidines, which include diminazene (Berenil) and pentami-

TABLE I. Infectivity of *Cryptobia salmositica* after in vitro exposure to isometamidium in whole fish blood.

Group	Treatment	Mean parasitemia (parasites/ml)	Fish with detectable infection
F	Infected controls	846,250† (137,500)	10/10
G	5×10^{-6} μg/ml*	139,063 (45,814)	9/10
H	5×10^{-5} μg/ml	40,278 (14,487)	8/10
I	5×10^{-4} μg/ml	5,001 (907)	5/10

* Concentration of isometamidium.
† Parasitemia and standard deviation () in juvenile *Oncorhynchus mykiss* at 3 wk after infection.

TABLE II. Effects of serum protein on the *in vitro* cryptobiacidal activity of isometamidium.

Treatment	Number of *Cryptobia salmositica**											
	3 hr			24 hr			48 hr			1 wk		
	A	B	C	A	B	C	A	B	C	A	B	C
Isometamidium† ± MEM with FBS	+	+	+	+	+	+	+	+	+	−	−	+
Isometamidium + MEM without FBS	−	−	−	−	−	−	−	−	−	−	−	−
Isometamidium + CBVR	−	−	−	−	−	−	−	−	−	−	−	−
Isometamidium + plasma	+	+	+	+	+	+	+	+	+	−	−	+
Isometamidium + whole blood	+	+	+	+	+	+	+	+	+	−	−	+
Chelated plasma	−	−	−	−	−	−	−	−	−	−	−	−
Plasma	+	+	+	+	+	+	+	+	+	+	+	+
CBVR	+	+	+	+	+	+	+	+	+	−	−	−
MEM	+	+	+	+	+	+	+	+	+	+	+	+
Whole blood	+	+	+	+	+	+	+	+	+	+	+	+

* Number of *C. salmositica* inoculated; A = 1,000; B = 10,000; C = 100,000.
† Stock solution containing 5×10^{-5} μg/ml of isometamidium: + wells containing living *C. salmositica*; − wells without living *C. salmositica.*

dine (Shapiro, 1993). Berenil and pentamidine bind to the minor groove of A–T-rich sites with high DNA sequence specificity (Reinert, 1999). Isometamidium is produced by linking a portion of the Berenil molecule to an ethidium nucleus, and consequently it also disrupts kinetoplast DNA (Kinabo and Bogan, 1988).

The mechanisms of action of drugs against trypanosomes are generally not well understood, with the exception of eflornithine (Wang, 1995). Eflornithine is used to treat early and late stages of *Trypanosoma brucei gambiense* infections and inhibits ornithine decarboxylase (Bey et al., 1987). Isometamidium inhibits both mammalian and trypanosomal DNA polymerases and interacts with glycosomes, kinetoplast DNA, glycoprotein biosynthesis, lipid metabolism, and membrane transport systems. It was suggested that these interactions may be part of the overall mode of action of the drug (Kinabo and Bogan, 1988). Several antitrypanosomal drugs are known to inhibit oxygen consumption, although this has not been demonstrated for isometamidium. For example, the two calcium channel antagonists, isradipine and lacidipine, inhibit growth and oxygen consumption of *Trypanosoma cruzi* epimastigotes at micromolar concentrations (Núñez-Vergera et al., 1998). Similarly, an antibiotic, ascofuranone, specifically inhibits respiration and in vitro growth of long slender bloodstream forms of *Trypansoma brucei brucei* (Minagawa et al., 1996). Licochalcone A alters the structure of the mitochondrion of *Leishmania* promastigotes and inhibits oxygen consumption and carbon dioxide production (Zhai et al., 1995). *Cryptobia salmositica* consumes oxygen under in vitro conditions (Thomas et al., 1992). The present study demonstrated that isometamidium inhibited oxygen consumption and carbon dioxide production by *C. salmositica.* Part of this could be due to its effects on the mitochondrion.

Oxygen consumption was inhibited by isometamidium and pyruvate and lactate increased after in vitro exposure to isometamidium. This may indicate that *C. salmositica* had switched to glycolysis after the mitochondrion was damaged. Electron microscopy of the mitochondrion showed a decatenation of kinetoplast DNA, vacuole formation, and a swelling of the cristae. However, the inhibition of mitochondrial dehydrogenases, i.e., lactate dehydrogenase, malate dehydrogenase, and succinate dehydrogenase, was not examined. Further studies on several enzymes of the electron transport chain in the mitochondrion are warranted to document further the exact mechanism of action of isometamidium on this organelle.

Trypanocides are classified into 2 broad categories based on the speed with which they lyse trypanosomes and their ability to affect the infectivity of trypanosomes (Hawking, 1963). The trivalent arsenicals, acriflavine and most diamidines, such as Berenil, are rapidly lethal and do not affect infectivity of trypanosomes. However, suramin, antrycide, and phenanthridinium compounds, such as isometamidium, take longer to kill trypanosomes but reduce infectivity. Similarly, the infectivity of *C. salmositica* in fish blood was reduced after in vitro exposure to isometamidium. However, this could also be because most *Cryptobia* were killed and therefore isometamidium would seem to reduce infectivity. The number of infected fish and parasitemias was lower in fish that received the parasites exposed to isometamidium (present study); this might be because these fish received lower inocula and also some of the parasites were not infective. Isometamidium at 1 ng/ml in Iscove's medium prevented infectivity of sensitive *T. brucei* stocks, *Trypanosome brucei evansi,* and *Trypanosoma vivax* (Kaminsky et al., 1990). Similarly, infectivity of *T. b. brucei* was prevented after incubation for 4 hr in plasma from cattle treated with diminazene aceturate.

Isometamidium is an amphiphilic cationic drug. It has many negatively charged groups and thus is strongly bound at physiologic pH by polyanionic molecules such as nucleic acids, mucopolysaccharides, and serum albumin. Isometamidium binds to serum proteins in rats (Philips et al., 1967), and this was confirmed in the present study. The lytic activity of isometamidium was significantly reduced in a medium containing serum supplement and this resulted in a delayed cryptobiacidal effect.

Ardelli and Woo (1999) demonstrated that 1.0 mg/kg isometamidium was therapeutic in rainbow trout during preclinical and chronic disease phases. However, the drug was not effective when trout were treated at acute disease, and as a result, the infection was more severe in treated as compared to nontreated fish. Two in vitro experiments were performed to examine the effects of isometamidium on complement-mediated lysis. The lytic titers of immune plasma or fresh complement did not change after 3 hr incubation with isometamidium (Ardelli and

Woo, 1999). Thus, isometamidium did not interfere with antibody production or function, or complement production, or ions required to initiate and regulate complement. Instead, it was suggested that isometamidium altered surface epitopes on *C. salmositica* and thus protected the parasite from complement-mediated lysis. This was confirmed in the present study as *C. salmositica* maintained in drug supplemented medium were not lysed by complement-fixing antibodies nor agglutinated by immune plasma.

Cryptobia salmositica has the first 5 enzymes of the Embden–Meyerhoff pathway, as well as the peroxisomal enzyme catalase, sequestered within glycosomes, and these enzymes were demonstrated in whole cell lysates of the parasite (Ardelli et al., 2000). The glycosome in *C. salmositica* may provide an advantage to the parasite by allowing the switch between glycosomal glycolysis in the absence of oxygen or inhibition of oxidative phosphorylation and mitochondrial respiration in the presence of oxygen. This may occur in the leech vector (hypoxic conditions) or after exposure to chemotherapeutic compounds, like isometamidium, that damage the mitochondrion.

ACKNOWLEDGMENTS

This study was supported by grants from the National Sciences and Engineering Council of Canada to P.T.K.W. Isometamidium chloride used in this study was donated by Andrew Peregrine (Department of Pathobiology, Ontario Veterinary College, University of Guelph).

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