RESEARCH ARTICLE

Wael Y. Attia

SCANNING ELECTRON MICROSCOPY ON THE EFFECT OF SODIUM HYPOCHLORITE ON ADULT SCHISTOSOMA MANSONI IN VITRO

ABSTRACT:
Sodium hypochlorite (NaOCl), electrochemically derived from sodium chloride solution, is the most convenient source of active oxygen. The high reactivity and non-specificity of NaOCl open ample scope for its wide medical application. The objective of this in vitro experiment is to examine the efficacy of NaOCl (1 and 10 ppm) against adult Schistosoma mansoni using scanning electron microscopy (SEM). The tolerance against NaOCl was determined by scoring the worm motility after 3, 6, 12 and 24 hours of incubation. In 1 ppm NaOCl-treated group, 60% of worms significantly collapsed and moved only parts of the body 3 hours post-incubation, 30% became immobile after 12 hours and 60% died after 24 hours. The motility of worms in 10 ppm NaOCl-treated group decreased with longer exposure, and all worms died after 24 hours of incubation. SEM revealed that NaOCl induced extensive tegumental damage. After treatment with 1 ppm of NaOCl, the male tegument showed slight to moderate peeling. There were also marked changes in the tubercles, where some were swollen and others showed shortening of the spines. A large number of blebs were observed on the surface of the tubercles, and some were disrupted. Tegumental damage became more apparent after treatment with 10 ppm of NaOCl, where it showed extensive peeling and erosion, and some of the tubercles showed shortening and complete loss of spines. The results suggest that NaOCl-induced lesions in the schistosome tegument may increase the tegument antigenicity through exposure of its surface antigens, allowing the host's immune system to function effectively against the worm. This may provide a further insight for rational design of novel complementary chemotherapeutic or vaccine strategies for the control of schistosomiasis.

KEY WORDS:
Adult Schistosoma mansoni, tegument, sodium hypochlorite, scanning electron microscopy.

INTRODUCTION:
Schistosomiasis is one of the most endemic health problems in the tropical and subtropical areas. It is estimated that 200 million people in the world are currently infected and about 600 million are at risk (WHO, 2002). Adult Schistosoma mansoni worms reside primarily in the mesenteric veins of the mammalian host and deposit massive egg concentrations in the vein lumen. The eggs pass into the host tissues where they cause hepatosplenic lesions, haematemesis and ascitis (van der Werf et al., 2002). Chemotherapy with oxamniquine and praziquantel is effective against adult worms and alleviates some disease symptoms. Reinfection is common, especially during childhood and adolescence (Wilkins, 1989), requiring frequent treatments with the potential to promote drug resistance (Cioli et al., 1995; Bennett et al., 1997) and often leading to severe clinical consequences (Montero and Ostrosky, 1997). Therefore, complementary approaches for the control of schistosomiasis are now envisaged (Tanner and Evans, 1994).

Sodium hypochlorite (NaOCl), electrochemically derived from aqueous sodium chloride solutions, is the most convenient and physiological source of active oxygen. It is nontoxic, safely used in drinking water (Khan et al., 2008), easily excreted, and has low molecular weight and small size which allow it to readily penetrate the cellular membranes (Eventov et al., 1998). NaOCl is well known as a potent disinfectant. Solution of NaOCl is effective in destroying the infectivity of a variety of viruses, bacteria and fungi (Gerba and Kennedy, 2007; Abadias et al., 2008; Peeters et al., 2008). In 1997, Al-Sharkawi suggested the use of chlorinated compounds, particularly NaOCl, as potent cercaricides. NaOCl was found to immediately suppress the infectivity of S. mansoni cercariae. The chlorinated compounds have the advantages of being far less expensive, and are accessible and affordable by almost all people residing rural areas.

Vaccination against human schistosomes is a major hope for effective control. Experimental trials in laboratory hosts are quite promising (McManus and Loukas, 2008; Bickle, 2009). The outermost surface of intra-
mammalian stages of the parasite, the tegument, is the key to the parasite’s success, but it is also generally viewed as the most susceptible target for vaccines. A number of different schistosome surface membrane antigens have been shown to confer partial protective immunity against challenge infection with schistosomiasis (Loukas et al., 2007). This is largely justified by the well established concept that tegumental components may play an important role in the host-parasite interaction (Skelly and Wilson, 2006). These surface membrane antigens are unusually diverse in their structure and stage specificity (Sher et al., 1989; Cesari et al., 2005). Interestingly, although all of the characterized vaccine molecules are situated in the tegument, their exposure on the parasite surface, in most instances, is transient (Lebens et al., 2004; McManus and Loukas, 2008); this would result in reduced surface antigenicity and the development of a tegument intrinsically resistant to immune damage (Skelly and Wilson, 2006).

Attempts to modify or expose the normally hidden schistosome tegument antigens may increase the tegument antigenicity, making it more accessible to the immune system of the host. The present study is conducted to evaluate the in vitro effects of NaOCl on adult S. mansoni worms using scanning electron microscopy, aiming to demonstrate the effect of this compound on the tegument of adult worms. This may provide a further insight for rational design of novel complementary chemotherapeutic or vaccine strategies for the control of schistosomiasis.

MATERIAL AND METHODS:
Preparation of NaOCl solutions:
Sodium hypochlorite (NaOCl; bleaching solution) produced by Egypt Corporation for Chemical Industries, El-Max, Alexandria, Egypt (active ingredient of approximately 5%) was used in this study. For preparation of the testing solutions, a stock solution of 100 ppm was prepared and concentrations of 1 and 10 ppm were prepared from the stock solution as previously described by Al-Sharkawi (1997).

Snails and cercariae:
S. mansoni-infected Biomphalaria alexandrina snails were obtained from Theodor Bilharz Institute, Imbaba, Giza, Egypt and maintained at the optimal laboratory conditions for several weeks. Cercariae were collected from infected snails by the method described by Liang et al. (1987) and were used within one hour of shedding.

Mice:
Adult male albino mice Mus musculus were bred at Theodor Bilharz Institute, Imbaba, Giza, Egypt. Six weeks-old mice, weighing between 16 and 22 g were used in this study. Animals were housed in cages and standard diet and tap water were available ad libitum. Room temperature was kept at 22-25°C and a 12:12 hour light-dark cycle was maintained throughout the experiment.

Experimental design:
Experimental infection was performed by body immersion of individual mice to a single dose of 100 S. mansoni cercariae as described by Liang et al. (1987). Two months post-infection, mice were sacrificed and all worms (male, female, or in coupla) were perfused from intestine, mesentery and liver using buffered saline solution according to the method of Liang et al. (1987). Worms were washed repeatedly in isotonic buffered saline and examined using a binocular microscope (X: 100) to collect intact and motile worms that were kept in culture medium 199 (M-5017, Sigma, USA Lot No. 053K83051) containing antibiotics (penicillin 50 IU/ml; streptomycin 50 μg/ml) as previously described by Jiraungkoorskul et al. (2006).

Three hundreds adult worms were randomly assigned to three equally sized groups: group 1 was the untreated control group; group 2 was treated with 1 ppm NaOCl; and group 3 was treated with 10 ppm NaOCl. An equal volume of a double strength concentration of hypochlorite was added to the culture medium to obtain the desired concentration. The same procedure was applied for control worms, using declorinated tap water instead of NaOCl solution. Worms were incubated in the culture medium at 5% CO2 and 37°C. After 3, 6, 12, and 24 hours of incubation, the percent motility was assessed by examination under an Olympus SZ-ST stereomicroscope (Tokyo, Japan).

Motility criteria:
Motility was scored using the following criteria: 3 (moving whole body), 2 (moving only parts of body), 1 (immobile but not dead, stained with vital dye), and 0 (immobile and dead, unstained with vital dye) (Jiraungkoorskul et al., 2006).

Statistics:
Statistical differences between the experimental groups were calculated using the Student’s t-test. P values less than 0.05 were considered to be significant.

Specimen preparation for scanning electron microscopy:
After 24 hours incubation period, worms were fixed in 2.5% glutaraldehyde-phosphate buffer (0.1 mol/L, pH 7.4) at 4°C for 24 hours and post-fixed in 1% osmium tetroxide for 1 hour. Specimens were washed in the same buffer, dehydrated in graded series of ethanol and dried using liquid carbon dioxide as a transitional medium. They were transferred on a specimen-holder and coated with a layer of gold in vacuum evaporator to prevent charging of the specimens. Worms were examined and photographed with a Camscan DV4-scanning
RESULTS:

Motility observation:

All worms of the untreated control group remained active, with whole body movement (score = 3) throughout the experiment. In 1 ppm NaOCl-treated group, 60% of worms significantly contracted and moved only parts of the body (score = 2) after 3 hours of incubation, 30% became immobile (score = 1) after 12 hours of incubation and 60% died (score = 0) after 24 hours of incubation. The motility of worms in 10 ppm NaOCl-treated group decreased with longer exposure. Ninety% of worms significantly contracted and moved only parts of the body (score = 2) after 3 hours of incubation, 50% of worms were immobile (score = 1) after 12 hours of incubation and all worms were dead (score = 0) after 24 hours of incubation (Table 1).

Table 1. Motility scores of control and NaOCl-treated S. mansoni worms at different hours post-incubation in vitro.

<table>
<thead>
<tr>
<th>Groups</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1 ppm</td>
<td>40</td>
<td>60*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 ppm</td>
<td>10</td>
<td>90*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Motility scores: 3, moving whole body; 2, moving only parts of body; 1, immobile but not dead (stained with vital dye); 0, immobile and dead (unstained with vital dye).

*: The mean difference was significant when compared with the control group (p < 0.05)

Untreated control worms:

Adult male S. mansoni worm is thicker than the female worm and had a longitudinal cleft, the gynaecophoral canal, in which the female is held during copulation (Fig. 1a). Both male and female worms have an oral and a ventral sucker situated near the anterior end (Fig. 1b). The major structural components of the dorsal surface of a male worm are the tubercles which protrude from the tegumental surface, which consists of a smooth wrinkled surface (Fig. 1c). Unique components of the tubercles are the small pointed spines located around the peak of the tubercles (Fig. 1d).

Effect of NaOCl (1 ppm):

Adult worms incubated in a medium containing 1 ppm NaOCl for 24 hours became contracted assuming a shrunken and tightly curved appearance (Fig. 2a). Higher magnification of the middle dorsal part of the male tegument revealed disruption of the normal tegumental architecture where the tegumental wrinkles became less pronounced with slight to moderate peeling of the tegument. There was also a change in the tubercles, where some were swollen and others showed shortening of the spines (Fig. 2b and c). A large number of blebs could be observed around and on the surface of the tubercles. Some of the blebs were disrupted (Fig. 2d&e).
Fig. 2. Scanning electron microscopic graphs of adult male and female *S. mansoni* treated with 1 ppm NaOCl. a) Male (M) and female (F) showing contracted and tightly curved appearance. b) and c) Middle dorsal part of male tegument showing a slight to moderate peeling (arrows). Tubercles (T) showed complete loss of spines (S). Few blebs (B) could be observed. d) and e) Higher magnification of the middle dorsal part of male tegument showing large number of blebs (B) around and on the surface of tubercles (T). Some of the blebs were disrupted.

**Effect of NaOCl (10 ppm):**

Adult worms incubated in a medium containing 10 ppm NaOCl for 24 hours became contracted and showed tightly curved appearance in a similar manner to those exposed to 1 ppm NaOCl (Fig. 3a). Male worms showed severe damage of the dorsal surface of the tegument. The most prominent change was extensive peeling and erosion. Few blebs could be observed on the surface of the tubercles (Fig. 3 b&c). Some tubercles showed shortening or complete loss of spines. Severe swelling accompanied by vacuolization and disruption of the blebs was also observed (Fig. 3 d&e).
DISCUSSION:

In the present study, the tolerance against NaOCl (1 and 10 ppm) was determined by scoring the worm motility after 3, 6, 12, and 24 hours of incubation. The motility of treated worms decreased with longer exposure and all worms were dead after 24 hours of incubation in 10 ppm NaOCl solution. These results are similar to those previously obtained with adult S. mansoni after in vitro treatment with praziquantel (William et al., 2001); with S. mansoni, S. japonicum, and S. haematobium after in vitro treatment with artemether and haemin (Xiao et al., 2001) and with adult S. mekongi treated with praziquantel and artesunate (Jiraungkoorskul et al., 2006).

NaOCl is well known as a potent disinfectant. Its low molecular weight and small size allows it to penetrate easily through the cellular membranes and hence can oxidize toxins that are present not only in blood, but also in tissues (Eventov et al., 1998). The effectiveness of NaOCl in disinfection processes depends on the available chlorine in the solution. Hypochlorous acid (HOCl) is a weak acid and dissociates to the hypochlorite ion (OCl-) and proton (H+) depending on the solution pH. It is generally believed that HOCl is the active species in the germicidal action (Fukuzaki, 2006). NaOCl showed deleterious effects against S. mansoni cercariae (Fripp et al., 1972). The survival of cercariae varied with the concentrations of NaOCl and the times of exposure (Al-Sharkawi, 1997). An ultrastructural investigation on S. monsoni cercariae showed that NaOCl caused loss of tail region, vacuolization of the tegument with subsequent disruption of the apical syncytial tegument layer, as well as changes in the head region (El-Shaikh, 2001). The mechanism of action by which NaOCl may affect the tegument of S. mansoni worms has not been clarified yet. However, NaOCl may cause biosynthetic alteration in cellular metabolism and phospholipid destruction (Guida, 2006); it may induce formation of chloramines which interferes in cellular metabolism (Hidalgo et al., 2002; Murina et al., 2006) or may cause an oxidative action with irreversible enzymatic inactivation, and a lipid and fatty acid degradation (Cortezzo et al., 2004; Small et al., 2007).

The tegument, which covers the entire surface of adult schistosomes, is a major interface between the parasite and its host. Since schistosomes can survive for years within the host bloodstream, they are clearly able to evade host immune responses, and their ability is dependent on the properties of the tegument surface (Skelly and Wilson, 2006). The damage to the tegument along the worm’s body could impair its function, so that;
it could easily be attacked by the host's immune system (Xiao et al., 2000). Lesions at the tegument seem to allow binding of antibodies to normally hidden parasite components (Brindley and Sher, 1987). Vacuolization of the tegument may be the direct effect of NaOCl on S. mansoni, but the mechanism by which these vacuoles are formed has not been clearly established. Shaw and Erasmus (1983) suggested that the vacuoles arise from dilation of the basal membrane, and they enlarge because of a water and ion imbalance. Moreover, it is believed that this imbalance may result in the impairment of transport function of the tegument leading to the surface swelling (Jiraungkoorskul et al., 2006). These effects could impair the function of the muscle and the tegument structure that result in the death of the parasite (Sobhon and Upatham, 1990). Moreover, spine destruction at the tubercles of NaOCl-treated S. mansoni is likely to involve its major constituent, actin, which is present in spines as tightly packed filament bundles (Cohen et al., 1982). The exposed actin on the surface spine may lead to binding of both anti-actin autoantibodies and actin-binding proteins (Linder and Thors, 1992).

In conclusion, this study showed that in vitro treatment of adult S. mansoni with NaOCl (1 and 10 ppm) caused contraction and decreased motor activity as little as 3 hours incubation, immobility 12 hours after exposure, and death after 24 hours. The tegument was severely damaged. The results suggest that NaOCl-induced lesions in the schistosome tegument may increase its antigenicity, and therefore, may be used to probe rodent responses to schistosome surface antigens. Whether this compound may be used alone or in combination with other anti-schistosomal drugs still need further studies.

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Dr. عتسا وي, تصوير الأثنين الإلكترون على تأثير هيبوريكت الصوديوم على دزان الدنار

الموضوع: المحموم

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