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# Inhibition of malaria infection and repellent effect against mosquitoes by chlorine dioxide

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**Abstract:** We examined whether chlorine dioxide ($ClO_2$) could inhibit malaria infections when applied to the skin. Anesthetized mice were divided into two groups: one group was sprayed with $ClO_2$ solution and the other with distilled water. Each mouse was then placed on a vessel housing malaria-infected mosquitoes. The proportion of mosquitoes that fed on mice sprayed with distilled water was 47.7% (42/88) and the proportion of malaria-infected mice was 54.5% (6/11). These values were 5.9% (6/101) ($p<0.01$) and 7.7% (1/13) ($p<0.05$) in $ClO_2$-sprayed mice. In a separate experiment, mosquitoes (*Anopheles stephensi, Culex pipiens pallens* and *Aedes albopictus*) were placed in a mesh-covered tube with an air-containing box at one end and a $ClO_2$-containing box at the other. Mosquitoes were more likely to stay at the side facing the air-containing box, which indicated that they avoided $ClO_2$ gas at concentrations as low as 0.03 ppm. $ClO_2$ spray and gas could be used as a mosquito-repellent and may also reduce the transmission of malaria.

Key words: $ClO_2$ gas, malaria, mosquitoes, repellent

## Introduction

Mosquitoes pose a great threat due to their potential transmission of infectious diseases such as malaria, dengue fever, yellow fever, West Nile fever, and virus-mediated encephalitis (Louis, 1999; Tu and Coates, 2004; Racloz et al., 2012; van den Hurk et al., 2012). A common route of malaria is through the bites of mosquitoes infected with *Plasmodium* species (Guilbride et al., 2012; Matsuoka et al., 2002). Thus, the best way to prevent malaria is to prevent mosquito bites. Several commercial repellents prevent mosquito bites, such as *N*,*N*-diethyl-*m*-toluamide (DEET), allethrin, and metofluthrin (Mafong and Kaplan, 1997). DEET is the world's most widely used insect repellent and is employed against a broad range of insects. However, concerns have been raised over the potential toxic effects of DEET on young children, pregnant women, and lactating women (Koren et al., 2003).

Chlorine dioxide ($ClO_2$), a water-soluble yellow gas, has been classified as a free radical due to the presence of an unpaired electron in its molecular orbital (Lynch et al., 1997). Presumably because of the nature of free radicals, $ClO_2$ exhibits strong oxidizing activity against various substances, such as proteins (Ogata, 2007), and many microbes, including the influenza virus (Ogata and Shibata, 2008; Ogata 2012). $ClO_2$ was shown to be effective in preventing influenza virus infections at a subtoxic concentration in animal experiments (Ogata and Shibata, 2008; Akamatsu et al., 2012). Very low concentrations of the gas were also shown to decrease absenteeism among school children by suppressing the occurrence of upper respiratory infections (Ogata and Shibata, 2009). Here, we examined whether an aqueous solution of $ClO_2$ available commercially in Japan as a household disinfectant could be used as a mosquito repellent. We showed that this spray could be used to prevent malaria infection by reducing mosquito bites because mosquitoes avoided $ClO_2$ gas in the ambient air surrounding the skin of $ClO_2$-sprayed mice.

## Materials and Methods

**Ethics statement.** Animal experiments were carried out in a humane manner after receiving approval from the Taiko Pharmaceutical Ethics Committee for Animal Experiments and the Institutional Animal Experiment Committee of Jichi Medical University. Experiments were conducted in accordance with Institutional Regulations for Animal Experiments and the Fundamental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Mouse.** Six-week-old female BALB/c mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). They were acclimatized for 1 week before experiments at 26°C with 60–70% relative humidity under a 13 h-light/11h-dark cycle.

**Mosquitoes.** *Anopheles stephensi* Liston, 1901 was obtained from the Imperial College London in 1992. *Culex pipiens pallens* Coquillett, 1898 and *Aedes*

*albopictus* (Skuse, 1895) were purchased from Sumika Technoservice Corporation (Takarazuka, Japan). Mosquitoes were kept in a laboratory at 26°C with 50–70% relative humidity under a 13 h light/11 h dark cycle and were fed via a sheet of filter paper soaked with an aqueous solution of 5% (w/v) fructose containing 0.05% (w/v) *p*-aminobenzoic acid (Nacalai Tesque, Kyoto, Japan) as described previously (Arai et al., 2004).

**Malaria parasite.** Malaria parasites (*Plasmodium berghei* Vincke & Lips, 1948) were obtained from the Imperial College London in 1992. They were kept at −80°C in 50% glycerol.

**Measurement of $ClO_2$ gas concentrations near the skin.** Mice were anesthetized with an intramuscular injection of pentobarbital sodium (1 mg in 50 microliter of saline). The abdomen was shaved with an electric shaver and sprayed 2–3 times with the $ClO_2$ solution (below) until the abdominal surface was slightly wet. The ambient $ClO_2$ gas concentration was measured at various times and distances from the abdomen using a $ClO_2$ gas analyzer (model 4330-SP, Interscan Corporation, Chatsworth, CA, USA).

**Preparation of infective mosquitoes.** Three mice were infected by an intra-peritoneal injection of mouse erythrocytes ($2\times10^6$) that had been infected beforehand with *P. berghei*. Approximately 2–5% of erythrocytes were infected after 3 days. Mice were then anesthetized with an intramuscular injection of pentobarbital sodium (1 mg). Three mosquito cages containing 80 to 120 female adults each were prepared. One infected mouse was placed in each cage. The mosquitoes were allowed to feed for 30 min at 20°C. Unfed mosquitoes were removed and fed mosquitoes were reared with sugar-soaked filter paper in a 20°C room. After 14 days, 10 mosquitoes were dissected and the midgut was observed. At least 6 among 10 mosquitoes developed oocysts in the midgut.

**$ClO_2$ spray and $ClO_2$ gas.** A commercially available aqueous solution of $ClO_2$ in the form of a spray (Cleverin S™, Taiko Pharmaceutical, Osaka, Japan) was purchased from a drug store. It contained 1.5 mmol/L of $ClO_2$. To prepare the $ClO_2$ gas, Cleverin G™ (Taiko Pharmaceutical), which evaporated $ClO_2$ gas constantly for more than one month, was used.

**Mouse infection experiments.** Mice were anesthetized as above and divided into two groups. The abdomen of each mouse was shaved with an electric shaver, and then sprayed as above with the $ClO_2$ solution or distilled water as a control. Prior to the experiment, 4–12 infective mosquitoes were transferred into plastic vessels with an inner diameter of 36 mm and a height of 65 mm. The vessels were covered with mesh. A single anesthetized mouse was placed on the top of each vessel so that the mosquitoes could feed freely under the mouse, but not escape from the plastic vessel (Fig. 1A). Mosquitoes were allowed to feed for 15 min, and the number that fed on blood was counted under a microscope. This experiment was performed three times.

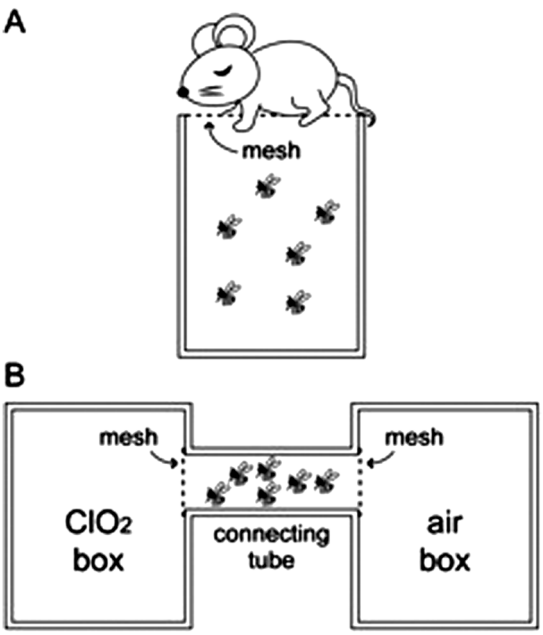


Fig. 1. Schematic representations of the experimental setups. (A) A mouse was anesthetized and its abdomen was shaved. It was then placed on a mesh-covered vessel containing 4–12 mosquitoes that had been infected beforehand with the malaria parasite. Mosquitoes were able to feed for 15 min. (B) Twenty mosquitoes were placed in a mesh-ended tube inserted into a box containing various concentrations of $ClO_2$ gas at one end and a box containing only air at the other. The dimensions shown are not exactly to scale.

**Demonstration of the repellent effect of $ClO_2$ gas.** Three species of mosquitoes (*An. stephensi, Cx. p. pallens*, and *Ae. albopictus*) were used for this experiment. Two plastic boxes (an "air box" and a "$ClO_2$ box"), each 30×30×30 cm, were connected with a hollow plastic tube with an inner diameter of 6 cm and a length of 40 cm (hereafter called the "connecting tube"). There was a hole with 10 mm in diameter in the middle of the connecting tube to insert mosquitoes. The tube faced the air box at one end and the $ClO_2$ box at the other. Each end was covered with mesh (Fig. 1B). The $ClO_2$ box contained various concentrations of $ClO_2$ gas in the air. The air box contained just air. Twenty mosquitoes were placed in the connecting tube through the 10 mm hole. The numbers of mosquitoes present at the sides of the tube were counted sixty seconds after they were placed in the connecting tube. The $ClO_2$ box and air box were exchanged each time to prevent the effect of light. Six experiments were performed for each concentration of $ClO_2$ gas.

**Statistical analysis.** Data were analyzed using the Student's *t*-test. The difference in rates between the two groups was evaluated by the chi-square test or Fisher's exact test. A $p$ value of less than 0.05 was considered to be significant.

Table 1. Number of mosquitoes that fed on mice and the number of mice infected with malaria.

| | $ClO_2$-sprayed mice | | | | Water-sprayed mice | | | |
|---|---|---|---|---|---|---|---|---|
| | Mouse | Malaria Infection | No. of mosquitoes that fed | No. of mosquitoes in vessel | Mouse | Malaria infection | No. of mosquitoes that fed | No. of mosquitoes in vessel |
| Exp 1 | 1a | + | 2 | 12 | 1e | — | 5 | 10 |
| | 1b | — | 2 | 12 | 1f | + | 6 | 12 |
| | 1c | — | 0 | 12 | 1g | + | 6 | 12 |
| | 1d | — | 2 | 12 | 1h | — | 5 | 12 |
| Exp 2 | 2a | — | 0 | 5 | 2f | — | 3 | 5 |
| | 2b | — | 0 | 5 | 2g | + | 3 | 5 |
| | 2c | — | 0 | 6 | 2h | + | 2 | 6 |
| | 2d | — | 0 | 6 | 2i | — | 4 | 6 |
| | 2e | — | 0 | 4 | | | | |
| Exp 3 | 3a | — | 0 | 6 | 3e | + | 3 | 7 |
| | 3b | — | 0 | 7 | 3f | — | 2 | 6 |
| | 3c | — | 0 | 7 | 3g | + | 3 | 7 |
| | 3d | — | 0 | 7 | | | | |
| Total | | 1/13 7.7%* | 6/101 5.9%** | | | 6/11 54.5% | 42/88 47.7% | |

* $p<0.05$ significant difference between the water- and $ClO_2$-sprayed groups.
** $p<0.01$ significant difference between water- and $ClO_2$-sprayed groups.

## Results

**Malaria infection of mice.** The number of mosquitoes that bit the mice, and the number of mice infected with the malaria parasite are shown in Table 1. When water was sprayed on mice, 42 of 88 mosquitoes fed on blood (47.7%) and 6 of 11 mice were infected with malaria (54.5%). In contrast, when $ClO_2$ was sprayed on mice, 6 of 101 mosquitoes fed on blood (5.9%) and 1 of 13 mice (7.7%) was infected. Significant differences were observed between the two groups.

**$ClO_2$ gas concentrations near mice sprayed with the $ClO_2$ solution.** Figure 2 shows the detection of $ClO_2$ gas after spraying the $ClO_2$ solution as a function of time and distance from the skin of these mice. The $ClO_2$ concentration increased at a distance of 3 cm from the abdomen after spraying and peaked (0.16 ppm) at five minutes. The concentration then decreased. The $ClO_2$ concentration was 0.04 ppm 30 minutes after spraying and then decreased to lower than 0.02 ppm. The $ClO_2$ concentration gradually increased at a distance of 50 cm from the abdomen and peaked (0.045 ppm) at ten minutes. It then gradually decreased.

**The repellent effect of $ClO_2$ gas against mosquitoes.** As shown in Table 2, mosquitoes clearly avoided the box containing $ClO_2$ gas, even at a concentration as low as 0.03 ppm. The number of *Ae. albopictus* mosquitoes at the "Air side" was significantly higher than that at the "$ClO_2$ side" ($P<0.001$), even at 0.02–0.03 ppm of $ClO_2$. The number of *An. stephensi* and *Cx. p. pallens* mosquitoes at the "Air side" was also significantly higher than that at the "$ClO_2$ side" ($P<0.05$), but was less than *Ae. albopictus* mosquitoes. Most of *Ae. albopictus* mosquitoes moved to the "Air side" at 0.10–0.12 ppm of $ClO_2$ concentration, however, half of *An. stephensi* mosquitoes and two thirds of *Cx. p. pallens* mosquitoes still stayed at the $ClO_2$ side.

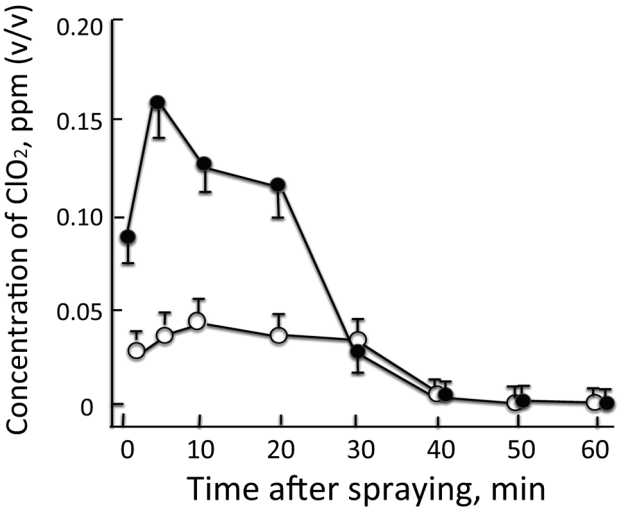


Fig. 2. $ClO_2$ concentrations in the ambient air around the skin of mice sprayed with a $ClO_2$ solution. Three mice were anesthetized and the abdomen was shaved. A $ClO_2$ aqueous solution was sprayed on the abdomen, and $ClO_2$ gas concentrations were measured for 60 min at distances of 3 cm (filled circles) and 50 cm (open circles) from the abdomen. Each point represents the mean for three mice with SD bars.

## Discussion

A lower number of mosquitoes bit mice sprayed with $ClO_2$ gas ($P<0.001$). In the water-sprayed group, 6 of 11 mice were infected with malaria (54.5%), while only 1 of 13 mice in $ClO_2$-sprayed group was infected (7.7%) ($P<0.05$). This preventive effect of the $ClO_2$ spray against malaria appears to have been due to the low rate

Table 2. Number of mosquitoes present in the connecting tube.

| $ClO_2$ ppm | Mosquitoes staying at the $ClO_2$ side/Air side | | | | | | Total (x/100) |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | |
| *Anopheles stephensi* | | | | | | | |
| 0–0.005 vs 0 | 10/10 | 9/11 | 9/11 | 11/9 | 10/10 | 11/9 | 60/60 (100/100) |
| 0.02–0.03 vs 0 | 7/13 | 6/14 | 7/13 | 8/12 | 6/14 | 8/12 | 42/78 (53.8/100)* |
| 0.05–0.07 vs 0 | 7/13 | 5/15 | 6/14 | 7/13 | 6/14 | 8/12 | 39/81 (48.1/100)** |
| 0.10–0.12 vs 0 | 5/15 | 6/14 | 5/15 | 4/16 | 4/16 | 6/14 | 30/90 (33.3/100)** |
| *Culex pipiens pallens* | | | | | | | |
| 0–0.005 vs 0 | 10/10 | 9/11 | 10/10 | 12/8 | 10/10 | 9/11 | 60/60 (100/100) |
| 0.02–0.03 vs 0 | 8/12 | 7/13 | 7/13 | 8/12 | 7/13 | 7/13 | 44/76 (57.9/100)* |
| 0.05–0.07 vs 0 | 6/14 | 8/12 | 7/13 | 5/15 | 6/14 | 6/14 | 38/82 (46.3/100)** |
| 0.10–0.12 vs 0 | 8/12 | 6/14 | 7/13 | 6/14 | 7/13 | 5/15 | 39/81 (48.1/100)** |
| *Aedes albopictus* | | | | | | | |
| 0–0.005 vs 0 | 11/9 | 10/10 | 11/9 | 8/12 | 10/10 | 9/11 | 59/61 (96.7/100) |
| 0.02–0.03 vs 0 | 4/16 | 5/15 | 7/13 | 5/15 | 5/15 | 6/14 | 32/88 (36.4/100)** |
| 0.05–0.07 vs 0 | 5/15 | 4/16 | 5/15 | 7/13 | 4/16 | 3/17 | 28/92 (30.4/100)** |
| 0.10–0.12 vs 0 | 2/18 | 1/19 | 3/17 | 3/17 | 4/16 | 2/18 | 15/105 (14.3/100)** |

* $p<0.05$ significant difference between the box with $ClO_2$ and air.
** $p<0.01$ significant difference between the box with $ClO_2$ and air.

of mosquito bites. The low rate of mosquito bites among $ClO_2$-sprayed mice may have been due to the repellent effect of the spray.

Therefore, we hypothesized that mosquitoes dislike $ClO_2$. Since $ClO_2$ is a water-soluble volatile gas, the ambient air near a $ClO_2$-sprayed mouse may contain an appreciable concentration of $ClO_2$ gas. To test this, the concentration of $ClO_2$ in the ambient air near $ClO_2$-sprayed mice was measured. Figure 2 shows the detection of $ClO_2$ gas after spraying the $ClO_2$ solution as a function of time and distance from the skin of mice. A gas concentration of over 0.03 ppm was measured at a distance of 3 cm from the abdomen, and this continued for 30 minutes. Taken together with the results in Table 2, mosquitoes may avoid coming to skin sprayed with $ClO_2$ for 30 minutes.

The proposed use of $ClO_2$ as a mosquito repellent may raise concerns regarding the potential toxicity of this chemical in humans. However, no exposure-related deaths were observed in rats following the administration of $ClO_2$ in drinking water over a 90-day period at a dosage level of 11.5 mg/kg body weight/day for males and 14.9 mg/kg body weight/day for females (Daniel et al., 1990). $ClO_2$ has long been used as a disinfectant in drinking water in some countries. The American Environmental Protection Agency (EPA) has stated that the "maximum residual disinfectant level" of $ClO_2$ in drinking water should be 0.8 mg/L (Environmental Protection Agency, 2002). Consequently, it is estimated that an individual may ingest 1.6 mg of $ClO_2$ per day, assuming he/she drinks 2 L of tap water per day. Regarding the use of $ClO_2$ in the form of a spray, information concerning dermal toxicity is extremely limited. An aqueous solution containing $ClO_2$ (9.7–11.4 mg/L) did not irritate the skin of mice over a 48-hour test period (Shi and Xie, 1999).

The results of the present study suggests that releasing a low concentration of $ClO_2$ gas into a room may prevent mosquito bites and, hence, mosquito-transmitted diseases. A $ClO_2$ gel (Cleverin G™) continuously evaporates $ClO_2$ gas into the surrounding environment and has been shown to maintain the concentration of $ClO_2$ at 0.03–0.10 ppm in a bedroom for more than one month. $ClO_2$ spray (Cleverin S™) on the skin may inhibit a mosquito's attack for 30 minutes. Further studies are needed to confirm that the topical use of DEET could be replaced by a low concentration ($< 0.1$ ppm) of $ClO_2$ gas.

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Conflicts of interest: Hiroyuki Matsuoka has no conflict of interest to declare. Norio Ogata is an employee of Taiko Pharmaceutical Co., Ltd.

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