

EFFECTS OF RIBAVIRIN AND HYDROXYUREA ON ORAL INFECTION OF *AEDES AEGYPTI* (L.) WITH DENGUE VIRUS

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Abstract. This study was conducted to determine the inhibitory effects of ribavirin and hydroxyurea on dengue virus replication in *Aedes aegypti* mosquitoes. Female *Ae. aegypti* mosquitoes were infected with dengue-2 virus and fed ribavirin at a dose of 0.3 mg/ml and/or hydroxyurea at a dose of 6 mg/ml via artificial membrane feeding technique. The virus in infected mosquitoes was isolated using C6/36 cell culture. Peroxidase-antiperoxidase (PAP) staining was used to detect dengue-infected C6/36 cells and to quantify the level of infection by determining the presence of infected cells. In mosquitoes treated with ribavirin alone, hydroxyurea alone or both drugs in combination had reductions in dengue infection rates of 87.72, 89.47 and 95.61%, respectively. The mortalities of female *Ae. aegypti* mosquitoes fed with these drugs were significantly higher than the control. Ribavirin also had an inhibitory effect on the fecundity of female *Ae. aegypti* mosquitoes.

Keywords: *Aedes aegypti*, inhibitory effect, ribavirin, hydroxyurea, dengue virus

INTRODUCTION

Dengue is today a serious mosquito-borne viral disease and is fast spreading globally (WHO, 2012). Two-fifths of the world's population (2.5 billion people) are estimated to be at risk for dengue infection (WHO, 2012). The World Health Organization estimates there are 50 million dengue infections worldwide every year (WHO, 2012). An estimated 500, 000 people with dengue hemorrhagic fever require hospitalization each year, a very proportion of whom are children (WHO,

2012). About 2.5% (25,000) of those die infected with dengue. Malaysia is no exception. Last year (2010) 46,171 cases were notified to the Ministry of Health and 134 deaths reported (Ministry of Health, Malaysia 2011). There is no specific treatment or effective vaccine against dengue. The control of dengue relies on the suppression and elimination of mosquito vectors. Conventional vector control is inadequate. Studies into new, effective means of control and treatment are urgently needed. We examined the inhibitory effects of antiviral chemotherapeutic agents, ribavirin and hydroxyurea, on the development of dengue virus in the vector, *Ae. aegypti*. The results may provides new avenues of interrupting transmission of dengue infection.

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MATERIALS AND METHODS

Mosquitoes

F 898 *Ae. aegypti* larvae were obtained from the insectarium of the Institute for Medical Research, Kuala Lumpur, Malaysia, and reared in white plastic trays (26cm x 36cm x 5cm). First and second instar larvae were fed with liver powder, while third and fourth instar larvae were fed with partially cooked liver. The emerged pupae were then transferred into plastic bowls which were placed in cages (30 cm x 30 cm x 30 cm) for emergence into adults. A cotton feeder fitted onto a test tube immersed in 10% sucrose solution with 1% vitamin B complex was placed in each cage.

Dengue virus

Dengue-2 isolated from human serum was obtained from Virology Unit of the Institute for Medical Research. The dengue-2 virus was maintained in cell culture in the laboratory.

Antiviral drugs

Ribavirin ($C_6H_{12}N_4O_5$) and hydroxyurea ($CH_4N_2O_2$) were obtained from SIGMA (St Louis, MO) and used at dosages of 300 g/ml and 6 mg/ml, respectively.

Artificial membrane feeding

When the mosquitoes were 5-6 days old, the sugar solution was removed and the mosquitoes were starved for one day. Female mosquitoes were removed from the cage using a battery operated aspirator and placed in paper cups in groups of 50 mosquitoes per cup. The paper cups were covered with nylon netting and secured with rubber bands. Fresh human blood, obtained 30 minutes before feeding, was mixed with the drugs and dengue virus. The feeding solution was standardized at a ratio of 67% blood, 25% virus and 8% sugar solution. The cups

were placed under a glass feeder in contact with the artificial membrane secured under the feeder. Feeding was conducted in an ACL-2 (Arthropod Containment Level-2) insectarium in accordance with biosafety procedures. The mosquitoes were allowed to feed for an hour as follows: C- (negative control, blood only), C+ (positive control; blood + virus), T1 (blood + ribavirin), T2 (blood + ribavirin + virus), T3 (blood + hydroxyurea), T4 (blood + hydroxyurea + virus), T5 (blood + hydroxyurea + ribavirin), T6 (blood + hydroxyurea + ribavirin + virus).

After feeding the mosquitoes were placed in 8 separate cages by group listed above. Normal saline was used to moisten the glass feeder after every feed. The circulating water was maintained at 37°C in a water bath and passed through the glass feeder via a water inlet and outlet to keep the blood warm. The mosquitoes were then kept in the ACL-2 insectarium with sugar solution and moistened filter paper allowing the mosquitoes to lay eggs for 7 days; the mortality of the mosquitoes was recorded. For each experiment, 3 replicates were conducted. The results were pooled and analyzed statistically.

Homogenate preparation

Surviving mosquitoes were removed and homogenized after being knocked down at -20°C. Each group of mosquitoes was placed inside a chilled eppendorf tube and then ground up with 1.5 ml of growth medium [Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS)]. The mosquito suspension was centrifuged at 3000 rpm for 15 minutes at 4°C.

Dengue virus isolation using *Aedes albopictus* clone C6/36 cells

Dengue virus was isolated using the method modified from Maneekam *et al*

Table 1
Effect of ribavirin (0.3 mg/ml) and hydroxyurea (6 mg/ml) on the mortality of female *Aedes aegypti* mosquitoes.

	Test group							
	C-	C+	T1	T2	T3	T4	T5	T6
Total number of mosquitoes	241	233	210	199	209	193	226	231
Total number of dead mosquitoes after 7 days	8	7	29	29	10	9	85	84
Mortality (%)	3.3	3.0	13.8	14.6	4.8	4.7	37.6	36.4
Compared with control (times)			4.2	4.9	1.5	1.6	11.4	12.1
p-value			0.001	0.001	0.43	0.37	0.001	0.001

C-, negative control; C+, positive control; T1, ribavirin; T2, ribavirin + dengue virus; T3, hydroxyurea; T4, hydroxyurea + dengue virus; T5, hydroxyurea + ribavirin; T6, hydroxyurea + ribavirin + dengue virus

(1993). One milliliter of each homogenate was passed through a 0.2–1 filter using a 2 ml syringe and then inoculated into a respective culture tube. The culture tubes were then vortexed and incubated for two hours at ambient temperature for absorption. Maintenance medium with 2% FCS was added and incubated at 28°C for 7 days.

Smear preparation

After 7 days incubation, cells from the sediment were transferred onto the Teflon-coated 12 well slides. The smears were left to dry at 28°C overnight in a laminar flow cabinet with the air blower on. The smears were then fixed with cold acetone for 20 minutes and then stained using the PAP method. Two smears were prepared for each experiment.

Peroxidase-antiperoxidase (PAP) staining

The PAP staining was performed as described previously by Igarashi (1982). The cells fixed with cold acetone were reacted with monoclonal antibody against dengue virus at a dilution of 1:5,000 at room temperature for 40 minutes, then rinsed in PBS and reacted with rabbit

anti-mouse IgG at a dilution of 1:1,000 for 40 minutes. They were then rinsed in PBS again and reacted with goat anti-rabbit IgG at a dilution of 1:1,000 for 40 minutes. After this, the cells were again rinsed in PBS and reacted with peroxidase-rabbit antiperoxidase complex at 1:1,000 for 40 minutes followed by washing and subjecting to peroxidase reaction for 5 minutes using 0.2 mg/ml of 3.3 diaminobenzidine (DAB) and 0.2% H₂O₂ as the substrate. The cells were observed under a normal compound light microscope at 400x. After staining, uninfected cells were colorless while infected cells stained red or pink.

Determination of dengue infection in mosquito

Dengue infection in the cultured cells was quantified by determining the number of positive fields per 100 fields examined. Two smear preparations were examined and the results were pooled and averaged.

RESULTS

Ae. aegypti feeding rates

The overall feeding rates of the mos-

Table 2
Effect of ribavirin/hydroxyurea on the fecundity of *Aedes aegypti*.

Replicates	Test group							
	C-	C+	T1	T2	T3	T4	T5	T6
Replicate 1	+	+	0	0	+	+	0	0
Replicate 2	+	+	0	0	+	+	0	0
Replicate 3	+	+	0	0	+	+	0	0

+, eggs laid; 0, no eggs laid

C-, negative control; C+, positive control; T1, ribavirin; T2, ribavirin + dengue virus; T3, hydroxyurea; T4, hydroxyurea + dengue virus; T5, hydroxyurea + ribavirin; T6, hydroxyurea + ribavirin + dengue virus

Table 3
Detection of dengue virus infection using cell culture and PAP staining.

Replicates	Smear	Number of positive fields/100 fields			
		Control	Ribavirin (0.3 mg/ml)	Hydroxyurea (6 mg/ml)	Ribavirin+ Hydroxyurea
Replicate 1	a	14	2	1	0
	b	24	3	3	0
Replicate 2	a	21	3	2	1
	b	17	1	2	1
Replicate 3	a	27	2	2	0
	b	11	3	2	3
Mean ± SD		19.00 ± 6.1	2.33 ± 0.8	2.00 ± 0.6	0.83 ± 1.2
Reduction (%)			87.79	89.47	95.63
p-value			0.001	0.001	0.001

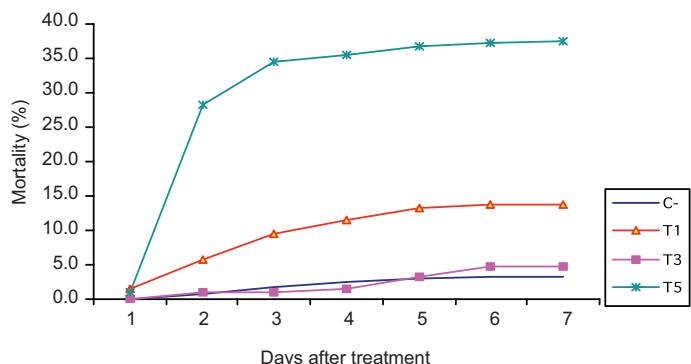
quitoes were 71.5, 71.4 and 74.9% for each replicate, with a mean of 72.6%.

Mortality rate of *Ae. aegypti* 7 days post-treatment

The effect of ribavirin (0.3 mg/ml) and hydroxyurea (6 mg/ml) on the *Ae. aegypti* mosquitoes (Table 1) was the mortality among mosquitoes treated with ribavirin was significantly higher than the control ($p<0.001$). The highest mortality was seen in the mosquitoes treated with a combination of ribavirin and hydroxyurea

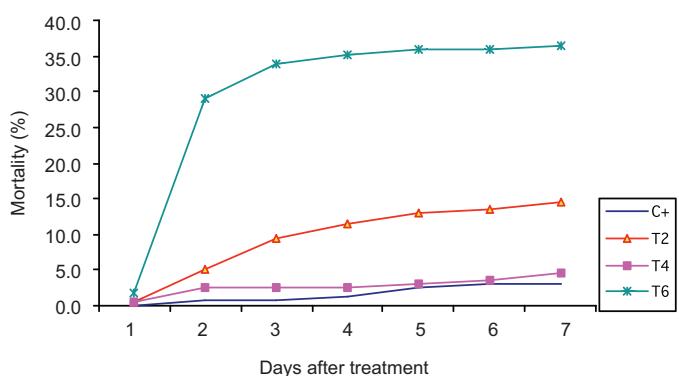
($p<0.001$). There was no difference in mortality between the mosquitoes treated with hydroxyurea and the corresponding control.

The daily mortality showed that the ribavirin combined with hydroxyurea induced a high daily mosquito mortality during the first 3 days of treatment (Figs 1 and 2). The mortality rates by the end of the first 3 days among the mosquitoes treated with overall mortality of mosquitoes treated with a combination



C-, negative control; T1, ribavirin;
T3, hydroxyurea; T5, hydroxyurea + ribavirin

Fig 1-Daily mortality of *Aedes aegypti* mosquitoes blood-fed with ribavirin and hydroxyurea.



C+, positive control; T2, ribavirin + dengue virus; T4, hydroxyurea + dengue virus; T6, hydroxyurea + ribavirin + dengue virus

Fig 2-Daily mortality of *Aedes aegypti* mosquitoes blood-fed with ribavirin, hydroxyurea and dengue virus.

of ribavirin and hydroxyurea, ribavirin alone and hydroxyurea alone, were 92.3, 67.2 and 36.8% respectively. There were no differences in mortality between dengue infected and uninfected mosquitoes.

Effect on fecundity of *Ae. aegypti*

All 3 replicates showed ribavirin exhibited a high inhibitory effect on

fecundity (Table 2).

Detection of dengue virus

After 7 days of tissue culture, the cells from each replicate were prepared in 2 smears (a and b) and PAP stained. Examination of infected cells was done using a normal light microscope (400x); infected cells stained red/pink. No infected cells were observed in the negative controls or uninfected tests. Table 3 shows the result of positive controls and those infected with dengue virus.

Among positive controls, the mean number of positive fields per 100 fields was 19.00 ± 6.1 . The mean numbers of positive fields per 100 fields in the ribavirin alone, hydroxyurea alone and ribavirin/hydroxyurea combination groups were 2.33 ± 0.8 , 2.00 ± 0.6 and 0.83 ± 1.2 , respectively (Table 3).

There was a significant reduction in dengue virus infection rate among drug treatment groups compared to positive controls. The percentage reductions in dengue infection in the ribavirin alone, hydroxyurea alone and combination groups were 87.79% ($p=0.001$), 89.47% ($p=0.001$) and 95.63% ($p=0.001$), respectively (Table 3).

DISCUSSION

Ussery (1981) studied the effect of ribavirin on dengue type 1 virus replication in LLC-MK2 cells. Ribavirin at concentrations ranging from 30 to 100 g/ml significantly suppressed the production of dengue virus. Ribavirin appeared to act by inhibiting viral RNA synthesis. This study

provided the basis for selecting the test dose of ribavirin (0.3 mg/ml) in our study.

Pampiglione *et al* (1985) proposed that drugs such as avermectins may be useful for the control of human and animal vector-borne diseases, if administered to a vertebrate host. They could cause the death of the hematophagous arthropod vector after taking a single blood meal from the treated host. Lee and Eng (1994) also reported a similar adulticidal effect of ivermectin on *Mansonia uniformis* and *Ae. togovi* feeding on the drug. Thornly (1999) reported a mortality rate of 39.0% 7 days post-treatment among *Ae. aegypti* mosquitoes fed on ribavirin at a dose of 0.3 mg/ml. Although hydroxyurea alone did not induce a very high mortality rate, it enhanced the adulticidal effect of ribavirin by 2 folds compared to ribavirin alone (Table 1).

In addition to the adulticidal effect, ribavirin exerted a strong effect on the fecundity of *Ae. aegypti*. No oviposition was observed among mosquitoes treated with ribavirin in any of the 3 replicates. Ribavirin has produced significant embryocidal and/or teratogenic effects in all animal species studied so far (FDA, 2011); malformations of the skull, palate, eyes, jaw, limbs, skeleton, and gastrointestinal tract have been noted. Abnormalities in sperm were also observed in studies of mice designed to evaluate the time course and reversibility of ribavirin-induced testicular degeneration (FDA, 2011). The present study is the first report of impairment of fertility among female mosquitoes. This may be important information for finding new approaches to interrupting dengue transmission by *Ae. aegypti* in high dengue risk areas. Manthri *et al* (1988) suggested factors that have an effect on the digestion of a blood meal and the rate of reproduction, are particularly

important in considering prospects for control of hematophagous vectors.

Because transovarian transmission of dengue virus in *Ae. aegypti* can play an important role in dengue virus maintenance in the urban areas (Lee *et al*, 1997), these drugs may be considered as a novel approach to interrupt transovarian transmission in the vector population.

Forks *et al* (1991) found *Ae. aegypti* mosquitoes fed on the blood of rabbits injected with ivermectin (at a dose of 0.2 mg/kg body weight) exhibited reduced survival (75% on day 3) and egg production (number of eggs = 13.2% of control); however, the author suggested ivermectin might not be useful for suppression of dengue infection because only adulticidal activity was achieved, the dose were prohibitively high and the density dependent nature of *Ae. aegypti* larval.

Our findings showed both ribavirin and hydroxyurea exerted a significant effect on the development of dengue virus in *Ae. aegypti*. Ribavirin at a dose of 0.6 mg/ml totally eliminated the virus in *Ae. aegypti*. Thornly (1999) also found *Ae. aegypti* mosquitoes infected with dengue virus and treated with ribavirin (0.3 mg/ml) had a lower infection rate than those with virus only; he also found hydroxyurea (6 mg/ml) alone reduced virus development by 89.47% among *Ae. aegypti* mosquitoes. The greatest inhibitory effect against dengue virus among *Ae. aegypti* mosquitoes was seen in those treated with a combination of both drugs. The reduction rate (95.63%, $p=0.001$) indicates the 2 drugs have a synergistic inhibitory effect on dengue virus development *in vivo* in *Ae. aegypti* mosquitoes.

These results provide important baseline information for further investigations. Although effective, ribavirin and

hydroxyurea cannot be used due to their inherent toxicity. However, our findings demonstrate the feasibility of interrupting dengue transmission using chemotherapeutic agents because of their adulticidal effect, inhibitory effect on vector mosquito fecundity and the anti-viral effect against dengue virus among vector mosquitoes which feed on drug-treated patients.

ACKNOWLEDGEMENTS

The authors thank the Director General of Health, Malaysia for permission to publish this paper, the Director of the Institute for Medical Research, Kuala Lumpur for support and the staff of the Medical Entomology Unit, IMR for help rendered. This study was partially supported by the National SEAMEO-TROPMED Centre, Malaysia.

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