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In vitro and *In vivo* Anti-Filarial Effect of Tetracycline/ Doxycycline

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Tetracycline is an established antibiotic and is targets to *Wolbachia* endosymbiont present within the filarial worms. Antibiotic treatment of filarial nematodes results in sterility and inhibits larval development and adult worm viability; this is due to effect on the *Wolbachia.* In the present study *in vitro* and *in vivo* anti-filarial effect of different concentration of tetracycline/ doxycycline was determined. *In vitro* results indicated that 50 µg/ml and 100 µg/ ml tetracycline concentrations affects the motility in both microfilariae and adult worms of *B. malayi*. The loss of motility was efficiently higher in female adult worms than male adult worms, could be attributed to the differences in *Wolbachia* load of the filarial worms. 14 (10 Male and 4 female) microfilaraemic subjects were treated with doxycycline @ 200mg/day for 30 days to study the *in vivo* filaricidal effect. In which the mean mf counts were gradually decreased after doxycycline treatment in both male and female subjects upto 6 months. The findings suggest that tetracycline/ doxycycline treatment can eliminate or reduce the mf load due to targeting *Wolbachia* endosymbiont therefore provide good tool for treatment and to hamper the transmission of filariasis from one host to another.

Keywords: *Wolbachia, Tetracycline, Doxycycline, in vitro,* Filariasis, *Brugia malayi*.

Lymphatic filariasis (LF) is a disease of considerable socio-economic burden in the tropics and sub-tropics and is caused by *Wuchereria bancrofti*, *Brugia malayi* and *B. temori*. *Wolbachia* endosymbiontic bacteria are found in mutualistic relationship in many filarial nematodes infecting animals and humans including *W. bancrofti*. Currently, the estimated 68 million people infected, among them, 36 million are microfilaria carriers and 40 million are symptomatic[1]. Additionally, 946 million people live in areas of southeast Asia and sub-Saharan Africa are at risk of infection[1].

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Anti-filarial chemotherapy is associated with systemic adverse reaction, due to release of microfilariae and *Wolbachia* bacteria into the blood. The available standard chemotherapy kills only the microfilariae and their macrofilaricidal function is not established either *in vitro* or *in vivo*. In recent past anti-*Wolbachia* antibiotics treatment has become a novel approach to treat lymphatic filariasis; these antibiotics inhibit worm development, embryogenesis, fertility and viability. The studies have demonstrated sub-lethal effect of antibiotics on filarial worms and this is due to effect on the *Wolbachia*[2,3,4]. Anti-*Wolbachia* treatment studies of tetracycline on animal models revealed the reduction in worm burden and blocking molting of infective stage larvae (L3 to L4 and L4 to L5)

as well as causes distortion of male/ female sex ratio[5,6,7].

Studies carried out in human subjects for the microfilaricidal and adulticidal effect of doxycycline showed significant reduction in microfilaraemia and antigenaemia after 8 weeks doxycycline treatment[8,9,10,11]. It has been shown that doxycycline treated female worm are sterilized for long term even permanent sterilization and majority of worms (90% in LF) are died[4]. The *in vitro* studies also demonstrated that antibiotics such as doxycycline, oxytetracycline and rifampicin have good activity against *Wolbachia*[12,13]. Thus different studies conducted on anti-*Wolbachia* treatment *in vivo* (animal and human) and *in vitro* demonstrated lethal effects on filarial parasite viability. The present study aimed to investigate the microfilaricidal and macrofilaricidal effect of tetracycline/ doxycycline *in vitro/ in vivo*.

MATERIALS AND METHODS

In vitro anti-filarial studies

Microfilariae (mf) were obtained from the peritoneal cavity and adult worm collected from the lymphatic tissue of infected *Mastomys natalensis*. Microfilariae and adult worm were washed with RPMI 1640 medium, plated on sterile plastic Petri-plates and incubated at 37°C for 1 h to remove *Mastomys natalensis* peritoneal exudates cells. The mf were collected from Petri-plates, washed with RPMI-1640 medium and used for *in vitro* maintenance. The collected mf separated by nuclepore filtration.

The effect of tetracycline on motility of *B. malayi* mf and adult was carried out in RPMI 1640 medium containing 5% FCS, 2mM L-glutamine, 100µg/ml streptomycin and 100U/ml penicillin. The mf (4000-5000 per well) and L3 (1-5 worms) were distributed in 6-well plate containing various concentrations of tetracycline (10 ¼g/ml, 20 ¼g/ml, 50 ¼g/ml, 100 ¼g/ml) for mf and (20 ¼g/ml, 50 ¼g/ml, 100 ¼g/ml) for L3 respectively in RPMI-1640 medium along with control (without tetracycline) and the motility was recorded at define time intervals for mf (upto 249h) and adults (upto 76h) after treatment.

In vivo anti-filarial studies

The study was carried out after taking information consent from filarial and healthy normal subjects residing in Raipur city, Chhattisgarh, India. A total of 14 microfilaraemic subjects (10 male and 4 female) age group of 14 to 62 were selected for the study. The range mf in 20 µl blood sample in male was 8-17 and in female was 2-40 recorded. The study protocols as well as the use of biological samples from human subjects were approved by the Ethical committee of Jiwaji University, Gwalior, (M.P). The Doxycycline was distributed to selected subjects at a dose of 200 mg/day for 30 days. Before and after treatment a 20 µl finger prick blood samples in duplicate were drawn from each subject between 20:00 to 22:00 hours. Thick smears were made and stained with Giemsa stain. The stained smears were examined under the microscope for mf count.

The adulticidal function of doxycycline was assessed by monitoring the adult movement of *W. bancrofti* in lymph nodes of various organs of the microfilaraemic subjects and one untreated filarial human subject, by Droppler pulse Sonography.

RESULTS AND DISCUSSION

In vitro effect of tetracycline

Though the chemotherapy with DEC is a comparatively a cheaper and cost effective but an important drawback associated with DEC is its side effects. The adverse effect of DEC limits its total acceptance by the population thus there is a need to find out safe treatment for LF. The antibiotic tetracycline/ doxycycline target the filarial endosymbiont, *Wolbachia,* opened new vistas for anti-filarial therapy. The microfilaricidal as well as macrofilaricidal function of tetracycline has been demonstrated *in vitro*[6] and *in vivo* in animal and human subjects[14].

Here, the impact of tetracycline exposure at different concentration on mf was studied *in vitro* and loss of mf motility was recorded as drug effectiveness on mf. The results indicated that the loss of motility was very high at concentration of 100µg/ml and 50µg/ml in comparison to 20µg/ml, 10µg/ml and control group [Table 1]. The motility was completely lost with increased exposure time (144h and 214h) in 100µg/ml and 50µg/ml concentration treated mf group. There was no notable difference in the motility loss in 10µg/ml treated mf group and in control group. Therefore, it revealed that the drug tetracycline

is highly microfilaricidal in nature and the effect was found dose dependent. Previous *in vitro* and *in vivo* Anti-*Wolbachia* treatment studies demonstrated the lethal effects of tetracycline on filarial parasite viability[2,3,11]. Similarly the treatment with tetracycline effectively blocks embryo development in two filarial nematodes namely *B. pahangi* and *D. immitis*[13].

The *in vitro* effect of different tetracycline concentrations on adult worms was showed that loss of motility was higher in 100µg/ml and 50µg/ml at 36h after treatment [Table 2]. The sex wise analysis showed that the motility was remaining higher (51-75%) in male and lower (26-50%) in female at 36h of 100 µg/ml treatments. After 36 h the motility was gradually decreases in both male and female worms and is completely lost in female worms at 56h of exposure. But in male worms motility was remained upto 11-25% at 56h of exposure and remains the same at 76h of 100 µg/ml treatments. As the concentration of drug decreases the motility was inhibited both male and female but intensity was very low. In comparison to male, the motility of female adult worms was completely lost after 56h at 50 µg/ml tetracycline concentration. The loss of motility was efficiently higher in female adult worms than male adult worms at all tetracycline concentrations tested.

Table 1. Effect of tetracycline on the motility of microfilariae of Brugia malayi

Tetracycline conc. / ml	Period of exposure in hours														
	0h	36h	60h	84h	108h	120h	132h	144h	156h	168h	180h	192h	214h	234h	249h
100µg	4+	3+	2+	2+	1+	1+	1+	-	-	-	-	-	-	-	-
50µg	4+	4+	4+	4+	3+	2+	2+	1+	1+	1+	1+	+/-	-	-	-
20µg	4+	4+	4+	4+	4+	4+	3+	3+	3+	3+	3+	3+	2+	2+	2+
10 µg	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+	3+	3+
Control	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	3+

4+ = 76-100% motility, 3+ =51-75% motility, 2+ = 26-50 % motility, 1+ = 11-25% motility,
+/- = 1-10% motility, - =0 % motility

Table 2. Effect of tetracycline on motility of adult worms of *Brugia malayi*

Tetracycline conc. / ml	Sex	Period of exposure in hours			
		0 h	36h	56h	76h
100µg	Male	4+	3+	1+	1+
	Female	4+	2+	-	-
50µg	Male	4+	3+	1+	1+
	Female	4+	3+	+/-	-
20µg	Male	4+	4+	3+	3+
	Female	4+	3+	1+	+/-
Control	Male	4+	4+	3+	3+
	Female	4+	4+	3+	3+

4+ = 76-100% motility 3+ =51-75% motility
2+ = 26-50 % motility 1+ = 11-25% motility
+/- = 1-10% motility - =0 % motility

Table 3. Effect of Doxycycline therapy on microfilaraemic subjects

Observations	Group	Before treatment	After treatment				
			1 month	2 month	3 month	4 month	6 month
No. Positive for mf	Male	10	10	10	08	08	08
	Female	04	04	04	04	04	04
No. successfully treated	Male	10	10	10	10	10	10
	Female	04	04	04	04	04	04
Completely cured	Male	00	00	00	02	02	02
	Female	00	00	00	00	00	00
Mean Mf	Male	9.8	3.14	1.5	0.3	0.3	0.3
	Female	20	14.75	12.8	7.3	7.5	5.5

It was indicated that the effect of tetracycline on adult filarial worms was more rapid in comparison to its effect on mf and it was gender dependent also. Thus tetracycline is an established antibiotic and is targeted to *Wolbachia* bacteria present within the filarial worms[15]. The gender dependent difference in adult filaricidal effect of tetracycline could be attributed to the differences in *Wolbachia* load of the filarial worms. The findings of Foster *et al*[16] supported our observation that the female worms harbor much higher loads of *Wolbachia* in comparison to their male counter parts. The death of filarial worms following the elimination of *Wolbachia* indicates the inevitable association of these two organisms.

***In vivo* effect of Doxycycline**

Thirty days course of doxycycline (200mg/day) was given to 10 male and 04 female human subjects with mf count 8-17 in male and 2-40 in female and was monitored upto 6 months after treatment. The mean mf count before treatment was 9.8 in male and 20 in female and the mf count decreased substantially in both the groups one month after the start of treatment [Table 3]. In male subjects infection was reduced gradually upto 6 months and it was observed after one month of treatment. Two out of 10 male subjects were found to be negative for mf count from 3rd month of treatment and remained negative during whole observation period (6 month). The gradual decrease in mf count was also observed in case of female subjects [Table 3]. After one month of treatment 14.75 mean mf count was recorded and which was reduced upto 5.5 mean mf count at 6 months after treatment. It was observed that doxycycline treatment effectively reduced the mf load due to targeting endosymbiont *Wolbachia*. These observations are supported by the reports published on killing of *Wolbachia* by doxycycline and subsequent reduction in microfilaraemia[6,13,15,17]. A significant reduction as well as complete elimination of microfilaraemia at 12 months after 6 weeks doxycycline treatment was reported[8,10,11,18]. The double blind field trail of doxycycline (200 mg/ day) for 8 week treatment on infected individuals, almost completely eliminate microfilaraemia, which was sustained for at least 8 to 14 months after treatment[10]. The advantage of the study is that a shorter treatment time (4 weeks) provides a gradual reduction in the mf count. Treatment with doxycycline/ tetracycline proves a effective tool to check transmission of filariasis from one host to another by lowering the mf load in the peripheral blood of the vertebrate host.

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