

Genotoxic Effects of Potentized Homeopathic Medicines on *Plasmodium Berghei*

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Abstract

Homeopathic drugs have been evaluated for their suitable candidature as potent antimalarials. However, the exact mode of action, of these homeopathic remedies still remains a matter of conjecture. This study aims to evaluate the efficacy of potentized homeopathic antimalarials on the integrity of the DNA of *Plasmodium berghei* (ANKA strain), in erythrocytes of Swiss albino mice. Short term *in vitro* culture of *P. berghei* was done with 50 µl of homeopathic medicines i.e. china (Chin.), chelidonium (Chel.), arsenicum album (Ars.alb.) and malaria officinalis (Mal.off.). The treated cells were passed through comet assay using ethidium bromide staining. The extent of DNA damage was expressed in terms of % head-DNA, % tail DNA, tail length and Olive tail moment. DNA damage was analysed in CASP software. Present study revealed that the 30 C potency of above medicines are capable of causing considerable DNA damage in the parasite. Maximum damage was observed in Ars. 30 C (16.4 ± 1.6 % tail DNA) followed by Chin 30 C (13.3 ± 0.7% tail DNA). DNA damage was significantly very less in Mal off. 30 C (2.6 ± 2.0% tail DNA) and Chel. 30 C (2.39 ± 0.4% tail DNA). Although, the exact mode of action of these remedies still remains doubtful. It is hypothesized that certain genes are responsible for the therapeutic activity of homeopathic remedies. From the study we conclude that even highly diluted homeopathic medicines disturbs the integrity of DNA. Further studies are required to confirm the role of specific genes and their expression to know the exact mechanism of ultra-high diluted drugs.

Keywords: *Plasmodium berghei*; Homeopathy; Genotoxicity; Potency

Introduction

Despite being used for centuries against many acute/chronic ailments, the mechanism of action of these ultra-highly diluted remedies which are actually diluted beyond Avogadro's limit (6.023 × 10²³), are still unrevealed.

On the contrary, within a decade many preliminary studies were carried out to check antimalarial activities of various homeopathic formulations to establish the existence and efficacy of these medicines. Pre-clinical studies, were done with mother tincture of Cinchona (Chin.) and its 30 potency (Chin. 30 C) to combat rodent malaria parasite in *P. berghei*. This treatment cleared the parasite from circulation and increased survival rate (26.8 ± 1.2 days) of mice [1]. Chin. is known to cures low forms of, various kinds of remittent or intermittent, or malarial fevers and kills parasites. It is likely that both Cinchona and malaria share some common 'effector toxin' resulting in a similar illness. Homeopathic Cinchona (Chin.) by neutralizing this common effector toxin cures malaria [2].

In a similar study, mother tincture of *Chelidonium majus* (Chel.) and its potencies i.e. 6, 30 and 200 were found to exhibit considerable antiplasmodial activity against blood stage infection of *P. berghei* in mice and 30 C was reported to enhance the Mean Survival Time (MST) of mice [3]. *Chelidonium majus* extracts are used mainly in the therapy of biliary and hepatic dysfunctions and it protected hepatotoxicity and showed an established therapeutic safety [4].

Malaria officinalis/Malaria nosode 20 was also reported to possess considerable antimalarial potential with 87.02% chemosuppression against *P. berghei* infection in BALB/c mice [5]. Nosode is a homeopathic preparation of the secretion from the affected area/tissue. Arsenicum album (Ars. alb.) is derived from the metallic element arsenic. It is the sovereign remedy for the malarial cachexia and typho-malarial fevers (fevers resembling both typhoid and malaria) [6]. Ars. alb. has been reported to have antimalarial potential against *P. berghei* infection in both *in vivo* and *in vitro* studies [7].

Despite of so many latest developments in the field of homeopathic research there is certainly a missing link between the existence of active molecules in these ultra-high dilutions and its mode of action. According to a hypothesis given by Khuda-Bhuksh (2009), potentized homeopathic medicines are capable of acting by triggering the regulatory action of master genes which initiates a series of reactions, resulting in many cellular and subcellular changes [8]. The mode of action of homeopathic dilutions are still a darker area in homeopathic research. The present study holds the objective of finding the mechanism of action of these antimalarial drugs (Chin., Chel., Mal. off. and Ars. alb.) which have already reported for their strong antimalarial potentials. The effect of these drugs on DNA of the parasite will confirm or deny their role in killing of parasite clearance from the circulation.

Materials and Methods

Maintenance of the parasite strain

White Swiss albino mice, weighing 22-24 g, aged 4-6 weeks old, of either gender were used as experimental models. The mice were maintained on a standard pellet diet and water ad libitum. The treatment of mice were according to the animal ethical clearance committee guidelines (45/1999/CPCSEA). Thawed cryopreserved strain of *P. berghei* (ANKA) was inoculated in mice. A mouse was monitored till infection reached up to 15-20%. Infection was maintained by means of weekly intraperitoneal inoculation of 1 x 10⁶ infected red blood cells (RBCs) to naïve mice [9]. The parasitemia was checked by preparing Giemsa stained thin blood smears on slides following incision of the tail vein of the infected mice.

Experimental drugs

Homeopathic formulations of Chin., Chel., Ars. alb and Mal. off/ Malaria Co nosode, all in 30 C potency (Dr. Reckeweg and Co. GmbH D.64625, Bensheim, Germany) were used in the present study. 90% ethanol was used as placebo.

Short term *In Vitro* culture

Short-term *in vitro* culture of *P. berghei* blood stages was performed according to modified method of Trager and Jensen (1976) [10]. RPMI-1640 medium (Gibco) supplemented with 0.06% (w/v) HEPES, 5% (w/v) sodium bicarbonate; antibiotics was used as culture medium (pH 7.4). Ten percent (v/v) inactivated fetal calf serum (FCS) was added to the incomplete medium to prepare the complete medium.

One ml of the complete medium contained 50 µl of homeopathic drugs Chin., Chel., Ars., Mal. off. or 90% ethanol (placebo). Each drug was checked in duplicate in a 24-well microtiter plate. After shaking gently the titer plate, 0 h smears were prepared, and the culture plate was incubated at 37°C in a candle jar (5% CO₂, 17% O₂, 78% N₂) according to Trager and Jensen's (1976) method. For checking the schizont inhibition after 21 h of incubation, the smears from each well were prepared, fixed in methanol, and stained with Giemsa stain.

Determination of DNA damage by comet assay

DNA damage of *P. berghei* treated with different homeopathic drugs was assessed by single-cell gel electrophoresis (comet assay) following modified method of Singh et al. [11]. Different stages of *P. berghei* were obtained from culture with homeopathic drugs. Samples were washed with cold phosphate buffered saline (PBS, pH 7.2), at 5,000 rpm for 5 min at 4. Cell mixture was suspended in Comet LM Agarose (1% low temperature-melting agarose, Himedia, Gaithersburg, MD, USA) at a ratio of 1:10 (v/v), 25 µl of the cell suspension was immediately placed on Comet Slides. Electrophoresis was performed in 1 x TBE buffer at 25 V for 10 min, stained with Ethidium bromide (Himedia), and observed under phase contrast microscope.

DNA Damage Analysis

The images were used to estimate the DNA content of individual nuclei and to evaluate the degree of DNA damage representing the fraction of total DNA in the tail. Cells were assigned to five classes: 0 (<7% of the DNA in the tail undamaged) 1 (7 to15%); 2 (15 to 22); 3

(22 to 30); 4 (>50% maximum damaged) as per the intensity of damage accordingly.

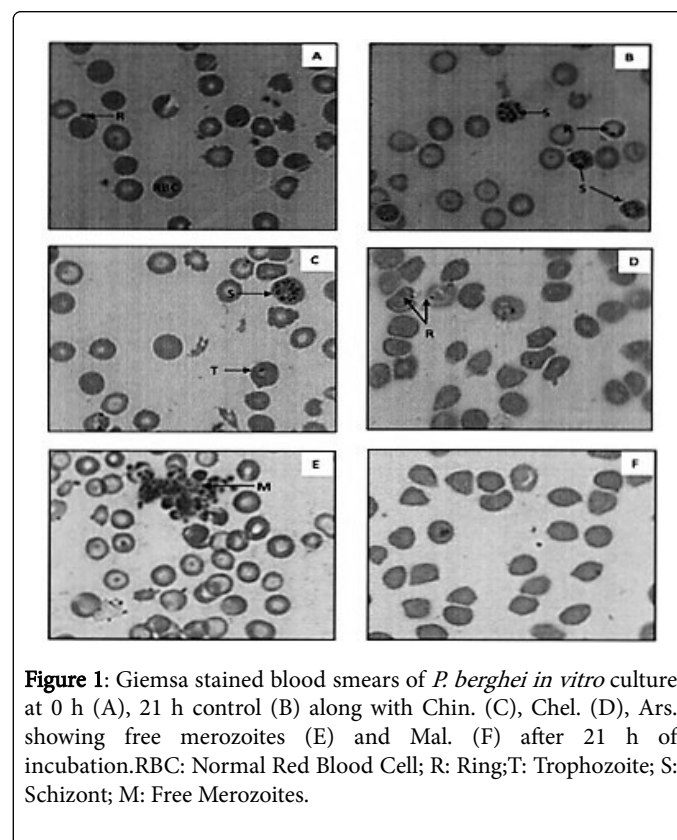
Data is presented as means ± SD. The parameters chosen for the quantification of DNA damage were: mean % Head DNA, tail intensity (% DNA) and tail moment as calculated by the CASP software.

Results

Short term *In Vitro* culture of *P. Berghei*

The short-term *in vitro* culture of *P. berghei* was done. Different stages of *P. berghei* from the culture were used for preparing samples for comet assay.

The lower layer of hematocrit consisting of rings and trophozoites were used for initiating culture. 1.6% infection was observed at 0 h (Figure 1A). After 21 h of incubation at 37°C, the culture was terminated and development of schizonts was checked. There was almost five fold increase in the parasitaemia (9.4%) after 21 h of incubation (Figures 1B-F). Smears were made from each well to confirm the presence of parasite. Cells from each well were taken and washed in prechilled PBS (pH 7.2) and used for comet assay.



DNA Damage Study

DNA damage levels (as a function of comet tail length) of *P. berghei* infected RBCs (IRBC) isolated from culture are shown in Figure 2A. The viability of *P. berghei* treated with different homeopathic formulations was >95% by ethidium bromide staining. As expected, the control and placebo samples didn't show any DNA damage. Whereas, considerable degree of DNA damage was observed in

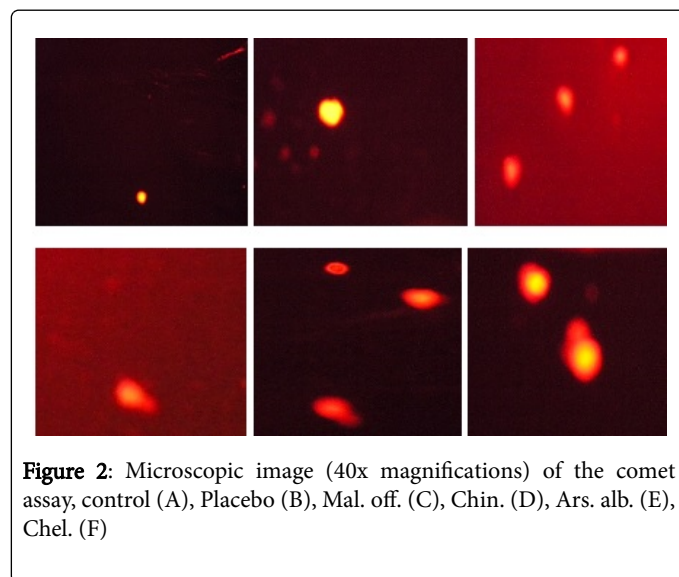
parasites treated with Ars. alb ($16.4 \pm 1.6\%$ tail DNA) followed by Chin. ($13.3 \pm 0.7\%$ tail DNA), Mal. off. ($2.6 \pm 2.0\%$ tail DNA) and Chel. (2.39 ± 0.4) (Table 1). Significant DNA migration/DNA damage were observed in them confirming that these homeopathic formulations caused genotoxicity to the parasite as analysed by the olive tail moment values (Table 1).

Maximum amount of damage was observed in parasites treated with Ars. alb. followed by Chin and the least damage was observed in Mal. off. treated samples.

Parameters	Groups					
	Control	Placebo	Chin.	Chel.	Ars. alb.	Mal. off.
L Head	173.2 ± 14.7	223 ± 12.2	103.2 ± 8.1	255 ± 10.8	157.1 ± 12.1	121 ± 9.4
L Tail	3.3 ± 0.2	3 ± 1.5	49.2 ± 2.1	21.2 ± 3.0	52.4 ± 4.9	14.7 ± 3.02
L Comet	176.3.2 ± 1.3	226 ± 6.5	152 ± 5.9	276.3 ± 8.3	209 ± 5.2	135.7 ± 4.6
Head DNA	99.9 ± 2.3	99.9 ± 1.3	86.6 ± 2.04	97.6 ± 3.1	83.55 ± 1.9	97.3 ± 2.8
Tail DNA	0.001 ± 0.1	0.0002 ± 0.002	13.3 ± 0.7	2.39 ± 0.4	16.4 ± 1.6	2.6 ± 2.0
TM*	3.2109 ± 1.06	7.221 ± 0.3	6.5 ± 1.5	0.50 ± 0.03	8.55 ± 1.1	0.36 ± 0.07
OTM#	0.0009 ± 0.0001	0.0002 ± 0.001	8.03 ± 1.1	2.41 ± 0.71	8.38 ± 2.02	1.59 ± 0.5

*Tail moment, #olive tail moment

Table 1: Showing length of head, tail, comet, amount of head DNA, tail DNA, tail moment and olive tail moment of cells treated with various homeopathic medicines. Analysed in CASP software.



In control and placebo treated groups the intensity of DNA damage in the cells were not seen and confirmed by the percentage tail DNA values i.e. $0.001 \pm 0.1\%$ and $0.0002 \pm 0.002\%$ respectively. As per the intensity of damage there was $<7\%$ of the DNA in the tail undamaged hence, the cells were visible as rounded/oval without any long tail (Figures 2A-2F). The above results confirmed that the DNA damage of parasite in treated groups was because of the homeopathic formulations and it did not showed any placebo effect.

Discussion

Biological activities of 30 C potency of Chin. 30, Chel. 30, Ars. 30 and Mal. off. 30 have been confirmed by preliminary laboratory studies

with respect to a multitude of scientifically accepted protocols, both in *in vivo* and *in vitro*. But there were no studies done to establish their mode of action. According to Khuda-Bhuksh, the well selected homeopathic remedies accelerate cellular and sub-cellular activities that are expected to be associated with a recovery process [8]. It is hypothesized that certain genes are responsible for the therapeutic activity of homeopathic remedies [12]. Using highly sophisticated molecular biological technologies, Sunila et al. [13] have reported in an *in vitro* setting that nosode carnosinum mother tincture do have the capacity to boost gene expression of anti cancer gene p53. Considerable evidence indicated that homeopathic remedies are capable of activating psycho-physiologic, self-regulatory mechanisms that mobilize hormones, neurotransmitters and components of immune system [14]. In case of homeopathic medicines, the stimulation of psycho-neuro-endocrino-immunological system with enzymatic system is the possible mechanism of action. From the previous studies it was clear that these drugs have strong antimalarial potential [5,15-17]. But, the mode of their antimalarial action was not clear.

Comet assay evaluates the shape of the DNA "comet" tail and migration pattern as an indication of DNA damage [18]. *P. berghei* infected RBCs from short term *in vitro* culture were recovered after 21 h *P. berghei* infected white swiss albino mice with 25-26% of parasitaemia was sacrificed for the culture. Presence of small but significant comet tail or DNA migration in all the treated samples clearly showed that the 30 C potency of homeopathic medicines Chin., Chel., Ars. alb and Mal off are genotoxic to the parasite. This indicated that DNA damage could be the reason for killing the parasites. Saha et al., 2013 also indicated that action of the potentized drugs are "more than placebo" and these ultra-highly diluted drugs are acted primarily through modulation of gene expression [19]. From the present results it's clear that these ultra high dilutions are capable of effecting DNA of parasite hence, it can be hypothesised that these formulations may

alter the expression of some genes controlling virulence and survival of the parasite as a result of which they were killed.

There is a possibility of some other mechanism by which the present drugs are interfering with the parasite and hampering its survival in the host. Lack of any conclusive evidence on the mechanism of action of homeopathy for most conditions calls for more clinical based studies. So, Homeopathy deserves an open-minded opportunity to demonstrate its value by using evidence-based principle. In future studies efforts will be made to check effect of these drugs on gene expression.

Conclusion

Present study reports the genotoxic nature of Chin. 30 C, Chel. 30 C, Ars alb 30 C and Mal off 30 C on the DNA of *P. berghei*. Presence of comet indicated damaged DNA in all the treated groups except placebo. We concluded that these homeopathic drugs were capable of killing the parasites by damaging their DNA but, there may be some other possible mechanism by which these drugs are disturbing survival of parasites and their virulence, which need to be found in future studies. Strong scientific basis will be needed to establish the mechanism of action of homeopathy.

Conflict of Interest

None.

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