The Bioscan 2 (1) : 15-20, 2007
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES

GENOTOXIC EFFECTS OF CARBARYL ON INDIAN CRICKET FROG *LIMNONECTES LIMNOCHARIS* OF WESTERN GHATS

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KEY WORDS
Genotoxicity
L. limnocharis
Chromosomal aberrations
Micronucleus assay
Sperm morphology assay

Received on :
19.10.2006
Accepted on :
31.01.2007

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ABSTRACT
Tracing many reasons, pesticidal cause is gaining momentum for the decline of amphibian population. As there are no reports on the effects of pesticides on amphibian fauna of Western Ghats, carbaryl a carbamate pesticide was used to test their genotoxic effect on *L. limnocharis*. The pilot toxicity studies have revealed that the LD_{50} was estimated to be 125mg/kg b. wt. at 96 h by an intraperitoneal injection. The median lethal and sub lethal doses 125, 93.75, 62.50 and 31.25 mg/kg body weight equivalent to LD_{50} and $3/4^{th}$, $1/2^{nd}$, $1/4^{th}$ of the LD_{50} were employed to treat the animals for further studies. Protocols such as chromosomal aberrations micronucleus test and sperm morphology assay were used to evaluate the genotoxicity. The frequency of chromosomal aberrations are not significant even at higher doses, micronucleus and sperm abnormalities were significantly more in higher doses of pesticide exposure during 72 and 96 h treated groups compared to controls.

INTRODUCTION

These days, pesticides are being widely used in agricultural fields to get rid of the insect pests rather to enhance agricultural production. These pesticides are known to cause deleterious effects when they enter the animal system. Of the three major groups of synthetic pesticides, carbamates are the recent additions. Many investigations have shown that some of the carbamate pesticides are mutagenic, clastogenic and carcinogenic. Even the cytotoxic effects of some of the carbamate pesticides have been reported.

Carbaryl is being extensively used in the paddy fields of Western Ghats, where *L. limnocharis* exists. No report is available on the effects of pesticide on this frog. Further, no cytological work has been done on this species. Cytological work is necessary to understand the chromosome complement of the species and in turn the cytogenetic effects. Hence, in the present investigation an attempt has been made to understand the effect of carbaryl on the chromosomes of bone marrow cells, micronucleus in erythrocytes and sperm abnormality in testes of *L. limnocharis*. The karyological and cytogenotoxic effects of carbaryl on the above species were undertaken and the results are presented in this paper.

MATERIALS AND METHODS

L. limnocharis were collected from Kuppalli Bioreserve Forest area (Western Ghats) and paddy fields of Lakkavally village (3 kms away from the University campus) and were introduced into artificial pond in the departmental premises to acclimatize to the laboratory conditions.(Fig.4)

Carbaryl (1-naphthal N-methyl carbamate) is used in control of insect pest. It is a broad spectrum carbamate insecticide, M/S Rallis India Ltd. Mumbai was the first to introduce it into the market in 1956 under the trade name of sevin in India.

The animals (1.8 to 2 gm weight 22-24mm length) were divided into 10 experimental groups, 1 group as control and 9 treated groups. In each group 10 frogs were introduced and various concentrations of pesticide was exposed for ten groups to find out the LD_{50} value at 96 h, on the basis of LD_{50} value of carbaryl, it's median lethal and sub lethal doses were fixed.

In the injection method, the known amount of pesticide was administered in to the animal body through an intraperitoneal injection by using 1ml syringe, only once in a treatment period. Based on the LD_{50} value, the animals were treated with doses. One median lethal (LD_{50}) and three sub lethal concentrations of $3/4^{th}$, $1/2^{nd}$ and $1/4^{th}$ of LD_{50} values of the carbaryl.

Preparation of chromosome plates

The colchicine of 0.006% concentration was used and the dosage of injection was fixed according to the body

weight of the animal (0.2 ml to 1.5 ml). After 14-16 h of administration of colchicine the animals were sacrificed

Chromosome plates were prepared from bone marrow cells of the frogs using conventional air dry technique (Evans *et al.,* 1964). Slides were stained with 0.5% Giemsa solution for 10-12 minutes and stained chromosomes in each treatment group about 400 well spread metaphase plates were screened for different types of chromosomal aberrations like chromatid breaks, a chromatid deletions, rings and minutes.

Karyotype: Karyotype was constructed according to Levan *et al.* (1964).

Micronucleus assay

For micronucleus assay, the animals were treated with different sublethal doses of carbaryl and controls were also maintained. The slides of the micronucleus assays were prepared by using the method of Schmid (1976). In each treatment group 20,000 erythrocytes (2000/slide) were scored for micronuclei by using Handtally counter.

Preparation of sperm smear

The abnormalities in the sperm morphology were studied in pesticide treated and control frogs using the technique developed by Wyrobeck and Bruce (1978). In each treatment group at least 20,000 sperms (2000/slide) were screened for the morphological abnormalities.

RESULTS

In the present investigation, we have noticed the karyotype of *L. limnocharis* (Fig.4) which comprised of 26 chromosomes (Fig.5 & 6) of which 5 pairs of large (Fig.6) and 8 pairs of medium to small chromosomes (Fig.6), of which 8 pairs were metacentric (1, 2, 4, 5, 6, 7, 12, and 13) and 5 pairs are sub metacentric (3, 8, 9,10 and 11),(Fig.5 & 6).

The median lethal and sub lethal doses of carbaryl have been treated to *L. limnocharis* and their effects on chromosomal aberrations were observed (Table 1, Fig.1). In all the four treatment groups 24 to 96 h time intervals resulted in frequency of chromosomal aberrations was increased, it was time and dose dependent manner. (Fig. 7, 11 &13)

The micronucleus test is another important tool employed for in in vivo cytogenotoxic studies to screen the clastogenic effect of the test chemical. Micronucleus were gradually increased and it was also time and dose dependent manner, were not significant at 24 and 48 h exposure, but whereas significant at 72 and 96 h when compared to control.(Fig.2, 8, 9 &10)

The frequency of sperm abnormalities was increased in pesticide exposed frogs and it was gradually time and dose dependent manner. Higher doses of the carbaryl at 72 to 96 h exposure were statistically significant when compared to control (Table 3 & Fig.3 & 11).

DISCUSSION

Our observations on the karyotype of *L. limnocharis* was

Table 1: Percentage frequency of chromosomal abnormalities in *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl

Treatment	Control	1/4th of LD_{50}	1/2nd of LD_{50}	3/4th of LD_{50}	LD_{50}
24h. M±SE	21±1.0	23.5±2.0	27±2.0	29.5±1	27.5±0.5
48h. M±SE	21±1.0	23±0	26±2.0	28±2.0	29.5±1.0
72h. M±SE	21±1.0	31±1.0	31.5±1.5	33±1.0	36.5±1.0
96h. M±SE	21±1.0	23±1.0	26.5±2.0	29±1.0	31.5±1.0

Table 2: Percentage frequency of micronucleus found in erythrocytes of *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl

Treatment	Control	1/4th of LD_{50}	1/2nd of LD_{50}	3/4th of LD_{50}	LD_{50}
24h. M±SE	0.05±0.009	0.07±0.009	0.06±0.008	0.08±0.008	0.07±0.019
48h. M±SE	0.05±0.009	0.11±0.01	0.12±0.014	0.125±0.017	0.13±0.022
72h. M±SE	0.05±0.009	0.15±0.008	0.21±0.023	0.2±0.017	0.25±0.022
96h. M±SE	0.05±0.009	0.13±0.008	0.15±0.019	0.14±0.012	0.185±0.025

Table 3: Percentage frequency of sperm abnormalities in *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl

Treatment	Control	1/4th of LD_{50}	1/2nd of LD_{50}	3/4th of LD_{50}	LD_{50}
24h. M±SE	3.08±0.08	3.21±0.184	3.24±0.185	3.23±0.184	3.33±0.167
48h. M±SE	3.08±0.08	3.01±0.176	3.07±0.08	3.3±0.05	3.5±0.828
72h. M±SE	3.08±0.08	3.42±0.173	3.53±.131	3.83±0.091	3.78±0.163
96h. M±SE	3.08±0.08	3.01±0.138	3.35±0.201	3.36±0.08	3.38±0.105

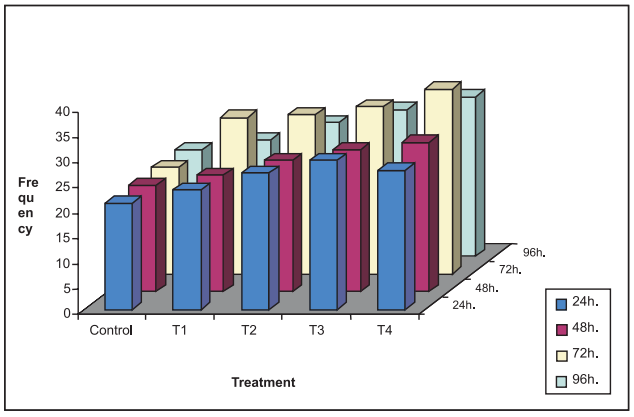

Figure 1: Percentage frequency of chromosomal abnormalities in *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl.

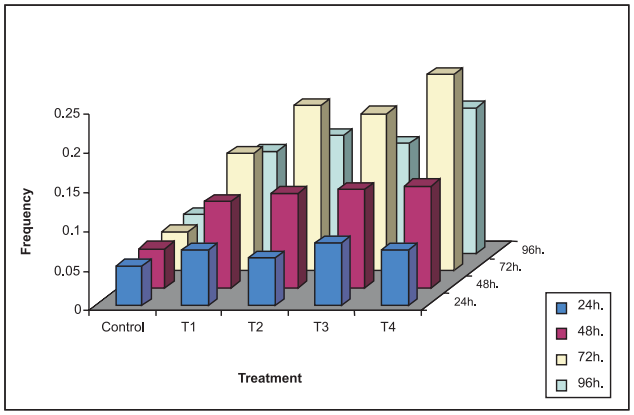

Figure 2: Percentage frequency of micronucleus found in erythrocytes of *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl.

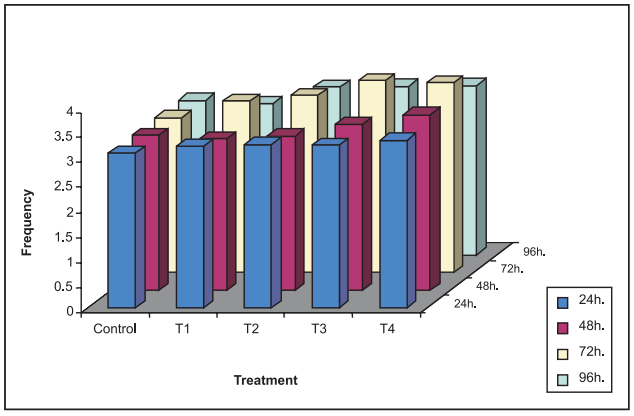

Figure 3: Percentage frequency of sperm abnormalities in *L. limnocharis* treated with median lethal and sub lethal doses of carbaryl.

Figure 4: *Limnonectes limnocharis*

similar to the observations of typical Ranid karyotype (King, 1990; Joshy *et al.*, 1999) and all previously examined species of the *Rhacophoridae* have 2n = 26 chromosomes (King, 1990; Kurmato, 1992; Prakash, 1998). The LD_{50} value of the carbaryl was 125mg/kg b.wt. at 96 .

Figure 5: Normal chromosomal plates

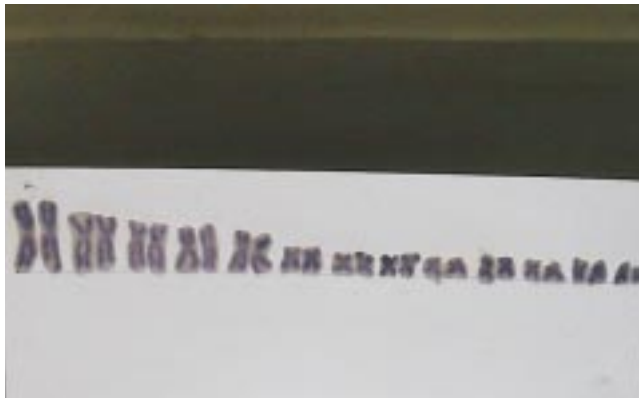

Figure 6: Karyotype of *Limnonectes limnocharis*

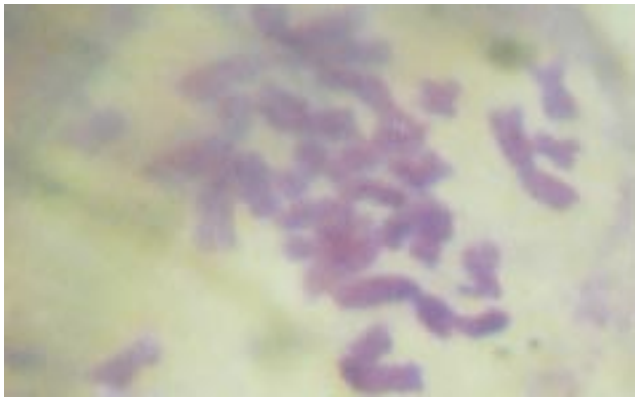

Figure 7: Chromatid deletion

The median lethal and sub lethal doses of carbaryl were 125, 93.75, 62.50 and 31.25 mg/kg b. wt. were treated to *L limnocharis* by an Injection method.

Increase of chromosomal aberration was evident at higher doses in all durations against control cells, which indicates clastogenic property of carbaryl. Similar to our present findings, in Chinese hamster cells, carbaryl exposure caused chromosomal aberrations (Onfelt and Klasterska, 1984). Carbaryl was mutagenic in a gene mutation assay with cultured Chinese hamster V79 cells, clastogenic in Chinese hamster fibroblasts induced chromosomal aberrations in

ovary cells and inhibited mitosis and spindle formation in cultured embryonic fibroblasts (Anonymous, 1987). It was reported that carbaryl depressed the mitotic activity of cells in onion root meristems (Sneizko *et al*., 1997; Nagpal *et al*., 1998). An oral administration of the carbofuran 1.9, 3.8 and 5.7mg/kg b.wt. for 4 consecutive days and observed chromosomal aberrations were significant to dose dependent manner (Chauan *et al*., 2000).

In earlier study, carbaryl showed a significantly higher

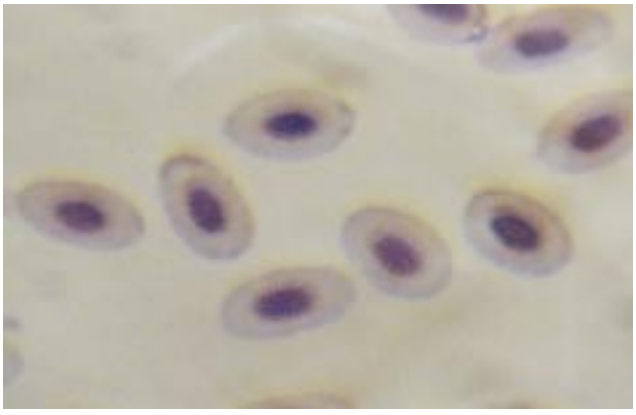

Figure 8: Micronuclei present in the erythrocytes

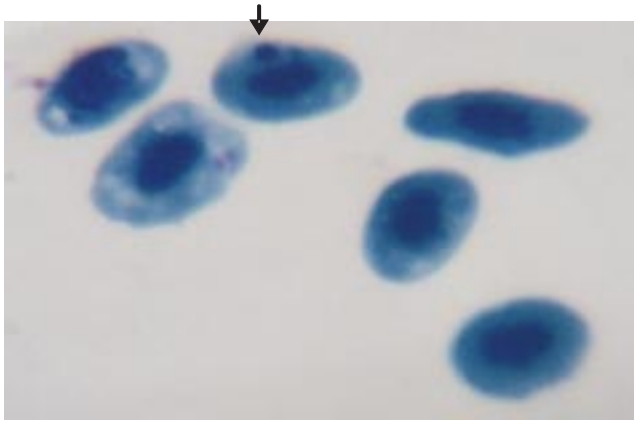

Figure 9: Normal erythrocytes

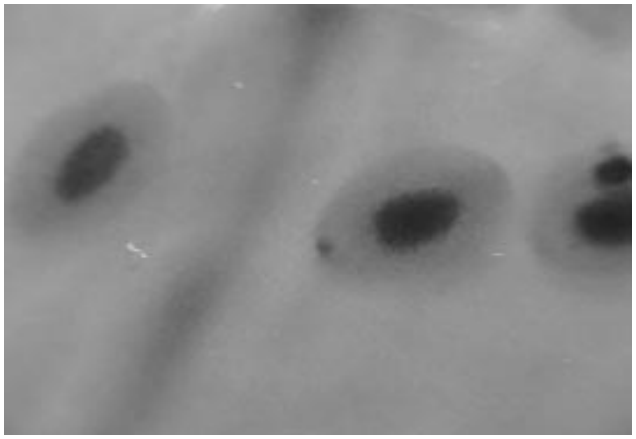

Figure 10: Micronuclei present in the erythrocytes

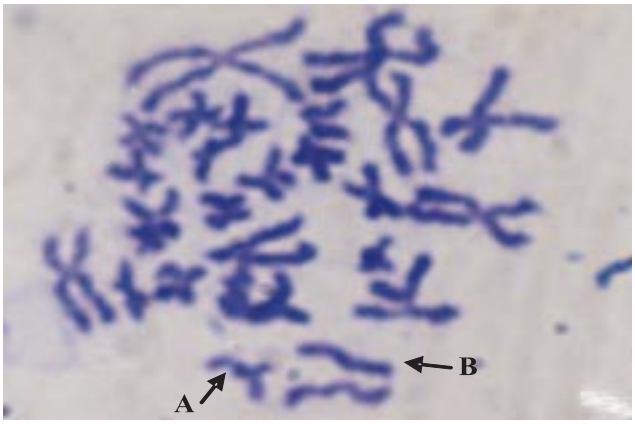

Figure 11: A. Duplication present in the chromosome and B. Breakage of chromosome

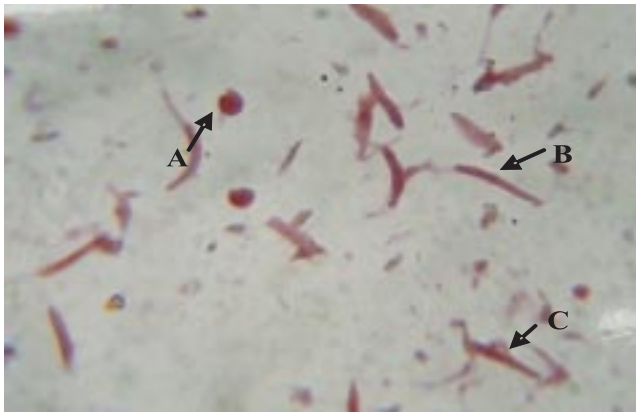

Figure 12: A. Sickle shaped sperm, B. Normal sperm and C. Hook shaped sperm.

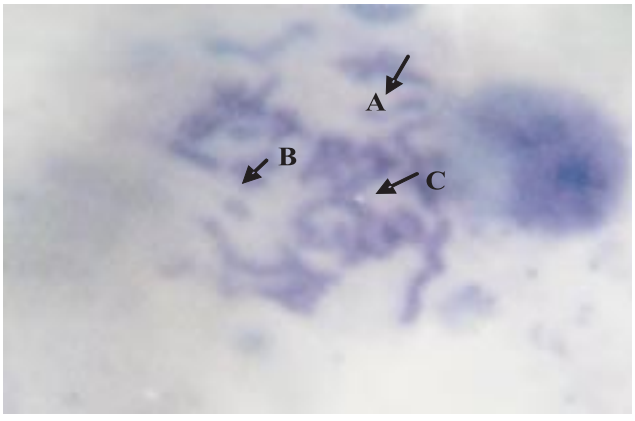

Figure 13: A. Deletions, B,. Minutes, C. Rings

frequency of chromosomal aberrations in kidney cells of fish *Channa punctatus*, indicates that carbaryl is a potent inducer of chromosomal aberrations (Sarangi, 2000). It was also reported that there was a significant decrease in mitotic index at higher doses of pesticide against control (Verma and Bharadwaj, 2003) indicates the mitotoxic property of carbaryl. In the present study, chromosomal

aberrations were increased in a time and dose dependent manner. Chromosomal aberrations in the entire treated groups were not significant when compared to control even at higher doses.

Increase of micronucleus was evident at higher doses in all durations against control cells, which indicates mitotoxic property of carbaryl. Earlier Marshal (1996) reported that carbaryl did not induce micronucleus at significant level in mice fed with carbaryl (50, 100 and 200mg/kg b.wt.) for 48 h. A similar result of Carbaryl (125 μg/l) induced micronucleus significantly at 25 days exposure in fish *Channa punctatus* was reported (Sarangi, 2000). In contrast, the results of the present study indicates that the carbaryl is an effective inducer of micronuclei but it induce significantly at 72 and 96 h when compared to control.

Increase of sperm abnormalities was evident at higher doses in all durations against control cells, which indicates genotoxic property of carbaryl. Carbaryl treatment on mouse in various doses upto 35 days did not show any significant increase in this sperm abnormality (Osterloh *et al*., 1983). An increase in sperm abnormalities in rats' fed with carbaryl for 60 days (Pant *et al.,* 1996) has been reported. Carbaryl induced acrosomal abnormalities in mice and human marginal or in conclusive effect was observed by Wyrobeck *et al*. (1983). Carbaryl has been reported to increase the proportion of abnormal sperm morphology in the pesticide exposed workers and reduce the sperm motility (ability to move) in rats (Shtenberg and Rybakova, 1968). Mice exposed to intraperitoneal dose of 1 and 2mg/kg b.wt. of carbofuran and repeatedly to 0.5mg/kg b.wt. for 5 consecutive days showed induced sperm abnormality significantly. It increased in dose dependent manner (Chauan *et al*., 2000). The results of the present study indicate that the carbaryl is an effective inducer of sperm abnormalities which is significant at 72 and 96 h.

Chromosomal aberrations induced in frogs by carbaryl exposure was not statistically significant even at higher doses. The micronucleus and sperm assay are, however, statistically significant at time and dose dependent manner, indicating the ability of carbaryl to induce micronucleus and sperm abnormalities.

ACKNOWLEDGEMENT

Supported by the SC/ST cell of the Kuvempu University for financial support and moral support by B.B.Hosetti, Reader, Dept. of Applied zoology Kuvempu University, Shankaraghatta, Shimoga.

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