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International Journal of Current Research
Vol. 4, Issue, 12, pp. 288-296, December, 2012

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

CHROMOSOMAL ABERRATIONS, MICRONUCLEUS AND SPERM MORPHOLOGY IN *Limnonectes keralensis* DUE TO CARBARYL

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ARTICLE INFO

Article History:
Received 18th September, 2012
Received in revised form
25th October, 2012
Accepted 19th November, 2012
Published online 18th December, 2012

Key words:
Frog- *L. keralensis*-carbaryl-Chromosomal aberrations-Micronucleus assay-sperm abnormality.

ABSTRACT

It is known that, the pesticide usages in agriculture have lead to increase in food production world wide. Due to agricultural managerial practices the anuran amphibians are much affected. In the present study, an attempt has been made on genotoxic effect carbaryl on *Limnonectes keralensis* in paddy fields of Central Western Ghats, where continuous application of pesticides is being practiced. Frog *L. keralensis* is a common inhabitant of such agro ecosystem. Carbaryl is a carbamate pesticide was used to test their genotoxic effect on *Limnonectes keralensis*. The pilot toxicity studies have revealed that LD_{50} was estimated to be 100mg/kg. b.w. at 96 h. by an intraperitoneal injection. The lethal and sublethal doses are 100, 75, 75, 50 and 25 mg/kg body weight. are equivalent to LD_{50} and 3/4th, 1/2nd and 1/4th of the LD_{50} doses were employed to treat animals for further studies. After treatment toxicity test like Chromosomal aberrations, Micronucleus and sperm abnormality were tested at 24, 48, 72 and 96 h duration of cabaryl exposure resulted in higher frequency of chromosomal aberrations when compared to control. The results reveal that the frequency of chromosomal aberrations, micronucleus and sperm abnormalities were not significant at 24 and 48 h at pesticide exposure, but significant statisticallay at 72 and 96 h in contrast to control. The pesticide causes these effects contribute to decline in its population too in long run.

INTRODUCTION

Chemical pesticides are well recognized in agricultural fields to control pests and to prevent the loss of agricultural production. At the same time pesticides are known to cause deleterious effects to other species in the environment. Of the three major groups of synthetic pesticides, carbamates are the recently introduced one. A number of studies were conducted on the toxicity of carbamate pesticides on various non target organisms and revealed that they are capable to bring mutagenic, clastogenic and carcinogenic changes. Even, some of the carbamate pesticides are potential to bring cytotoxic effects (U.S.EPA 1991; Caroline Cox 1993). N- methyl-1-naphthyl carbamate, Sevin has been reported to be responsible for the arrest of mitosis in roots of *Allium cepa* (Amer 1965). In comparative studies of three carbamate pesticides such as Dithane M-45, Baygon and Furadon on mice revealed that they are not effective in inducing statistically significant sperm abnormalities in mice (Vasudev and Krishnamurthy 1995). Anuran frogs are quite abundant, richly varied and wide spread in distribution. Frog diversity and their karyotypes make them particularly suited for cytogenetic studies. This investigation gives the first indications of the process that could have taken place during the course of chromosomal evolution in anurans and thereby degree of relatedness between individual species could be determined. While anthropogenic chemicals occur in a variety of ecological communities, ecologists have a poor understanding of their impacts on species within these communities. Aquatic communities in particular are commonly contaminated with chemicals including a wide variety of pesticides used to improve both agriculture and human health (McConnell 1998; LeNoir *et al*., 1999; Sparling *et al*. 2001; Kolpin *et al*., 2002 ; Fellers *et al*., 2004; Relyea 2006). Laboratory experiments have provided basic data about the effects of pesticides on model organisms, but ultimately we would like to know how pesticides affect a diversity of organisms under more natural conditions. Effects of carbaryl cause chromosomal aberrations in bone marrow cells of rat (Verma and Bharadwaj 2003).Carbaryl significantly induced high frequency micronuclei in bone marrow cells and peripheral erythrocytes of chick following intraperitoneal injection at 24 h (Bhunya and Jena 1995). Male and female rats were fed with carbaryl, it did not induce micronucleus significantly (Marshal 1996).

Aly (1998) has reported cytogenetic and genotoxic effects of carbofuran in adult mice using micronucleus assay. Carbaryl caused negative clastogenic response that was reported by Ma *et al*. (1984) who has used the plant Tradescantia for micronucleus test. In another study, carbaryl has caused significantly higher frequency of micronucleus in kidney cells of fish *Channa punctatus* indicating that carbaryl is a potent inducer of micronucleus (Pradipta Kumar Sarangi 2000). In 1980 US Environmental Protection Agency (EPA) review cited four studies (three in rat, one in another rodent) showing an increase in sperm abnormalities caused by carbaryl exposure. Carbaryl exposure caused low sperm count and abnormal sperm counts in human male workers (Cox 1993). It has also been reported that there was an increase in sperm head abnormalities in male rats' fed with carbaryl for 60 days (Pant *et al*., 1996). Male germ cells are crucial in the reproductive

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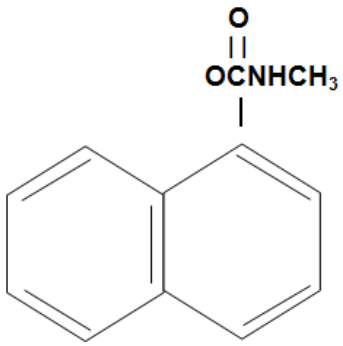
process and carbaryl exposure had strong correlation with low semen quality and sperm shape abnormalities in exposed employees (Jubler *et al*., 1999). Carbaryl induced sperm abnormalities positively in earthworm *Metaphire posthuma* (Gupta and Saxena 2003), but it did not induce sperm abnormalities significantly but increased DNA damage in exposed human beings (Meeker *et al*., 2004). Carbaryl is being extensively used in the paddy fields of Western Ghats, where *L. keralensis* exists. No report is available its effect of on this frog. Cytological work is necessary to understand the chromosome complement of the species and inturn to cytogenetic effects. Hence, the present investigations, dealt with the effect of carbaryl on the chromosomes of bone marrow cells, micronucleus in erythrocytes and sperm abnormality of *L. keralensis*. The karyological work was taken up first and later the cytogenotoxic effect of carbaryl on the above species was undertaken.

MATERIALS AND METHODS

About pesticide used

Carbaryl (L-Naphthyl N-methyl carbamate) is a broad-spectrum carbamate insecticide ,manufactured by M/s. Rallis India Ltd., Mumbai. This pesticide is inhibiting acetylcholine esterase enzyme. It is broad spectrum insecticide whose half life depends upon site conditions including sunlight and pH; for example, the half Life of carbaryl ranges from 0.1d at pH =8 to 1500d at pH <6 (Relyea, 2006).



Chemical Formula: $C_{12}H_{11}NO_2$

Test Animals

The Indian Verrucose frog (*L. keralensis*) is believed to be endemic to the Southern Western Ghats was selected as our test animal. According to IUCN criteria *L. keralensis* is in lower risk- near threatened (Daniels, 2005). *L. keralensis* is ochraceous black in colour, black markings were on back and limbs variable. A broad pale mid-dorsal stripe is frequently present (Fig 1). Specimens of *L. keralensis* were collected from Kuppalli Bioreserve forest area which is 100 km away from Kuvempu University (Central Western Ghats) and paddy fields of Lakkavally area which is 6 km away from Kuvempu University and were introduced into artificial pond in the departmental premises to acclimatize themselves to laboratory conditions for 10 to 12 days. The Kuvempu University animal ethical committee clearance has been obtained for performing then studies. After acclimatization the animals weighing in between 1.8 to 2 gms were divided into 10 experimental groups (1 control and 9 pesticide treated groups). In each group 10 frogs were introduced and they were subjected to expose to selected doses or pesticide.

Experimental protocol

In Injection method, the chemical was introduced to the animal body through injection by using 1ml syringe. LD_{50} value of carbaryl on *L. Keralensis* was 100mg /kg. b.w. at 96 h. Based on the LD_{50} value, the animals are treated with four different lethal and sub lethal doses. The sub lethal doses of carbaryl were $1/4^{th}$ of LD_{50} is 25mg/kg b.w. is considered as Group I or (GI), $1/2^{nd}$ of $LD_{50,}$ is 50mg/kg b.w. is considered as Group II or (GII), $3/4^{th}$ of LD_{50} is 75mg/kg b.w. is considered as Group III or (GIII), and lethal dose LD_{50} of is 100mg/kg. b.w. is considered as Group IV or (GIV), by Injection method. After treatment for every 24 h up to 96 h chromosomal aberrations, micronucleus and abnormal sperm effects were observed.

Preparation of Chromosome plates

0.006% concentration of colchicine (mitotic inhibitor) was used and the dosage of injection was fixed according to the body weight of the animal as (0.2 ml to 1.5 ml). Colchicine was administered intraperitoneally, after the 14-16 h of its administration the animals were sacrificed and used for further studies. Chromosome plates were prepared on slides from bone marrow cells of frogs using conventional air dry technique (Evans *et al*. 1964). The slides were stained with 0.5% Giemsa solution for 10-12 minutes and stained chromosomes in each treatment group 400 well spread metaphase plates were screened for different types of chromosomal aberrations.

Micronucleus assay

Micronucleus assay was analyzed in carbaryl treated and control frogs. 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.

Preparation of sperm smears

The abnormalities in the sperm morphology were also studied in carbaryl treated and compared with control frogs. The technique was used which was developed by Wyrobeck and Bruce (1978). For each treatment group at least 20,000 sperms (2000/slide) were screened for morphological abnormalities. Statistical analysis: The data was analyzed by Student‘t’ test using SPSS programme (Version 10.5) to derive the significance. The values were expressed as mean ±SE. The values obtained were expressed as percent change (+ or -) over the control.

Fig.1. ***Limnonectes keralensis***

RESULTS AND DISCUSSION

Chromosomal aberrations

The results indicate that total number of chromosomal breaks per 400 metaphase plates show gradual increase on pesticide dependent manner and exposure duration dependent manner. In all the four doses of treatment at 24, 48, 72 and 96hrs duration exposure resulted in higher frequency of chromosomal aberrations when compared to control (Table 1) (Fig.2,3ab,4,5ab,6abc,7 and 13).

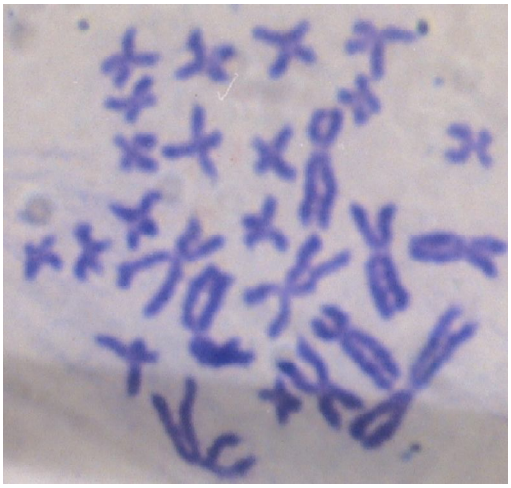


Fig 2. Normal chromosome plate

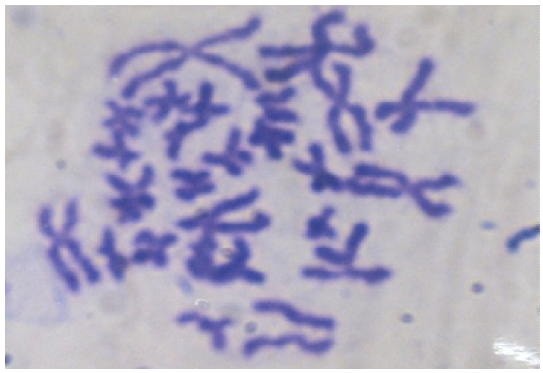


Fig. 3 a. Duplication of chromosome b. Breakage of the chromosome

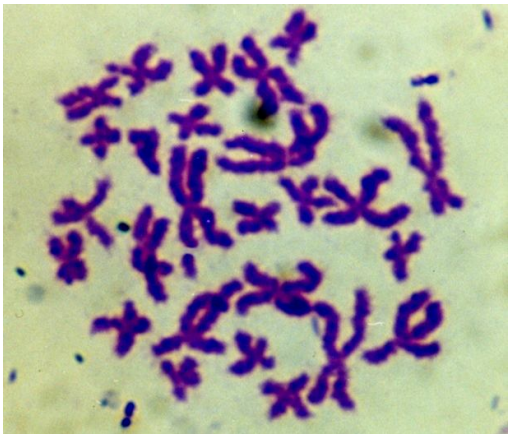


Fig-4. Chromatid aberration

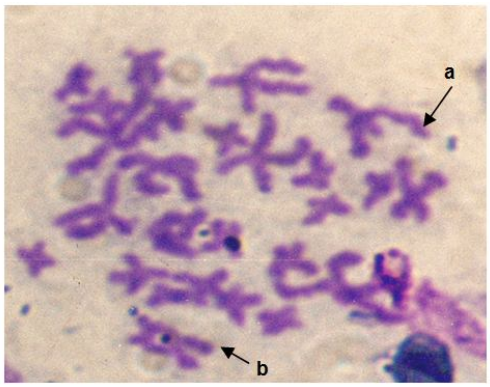


Fig. 5. a. Duplication of chromosome b. Dicentric chromosome

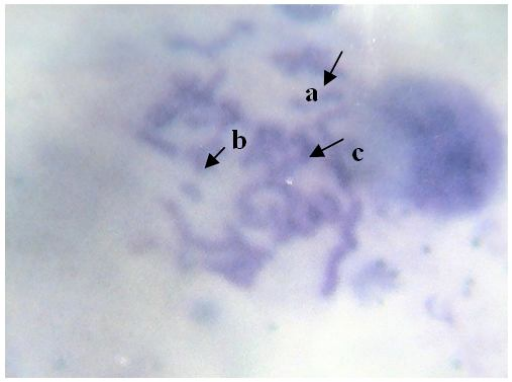


Fig.6. A. Deletion, b. Minutes, c. Rings

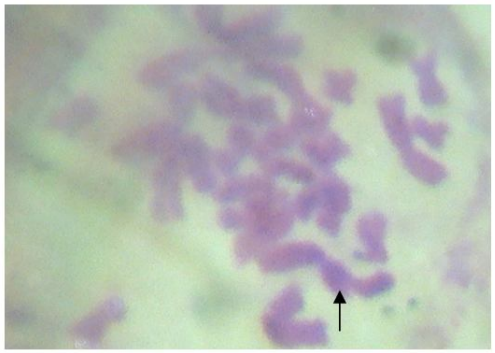


Fig. 7.Chromatid deletion

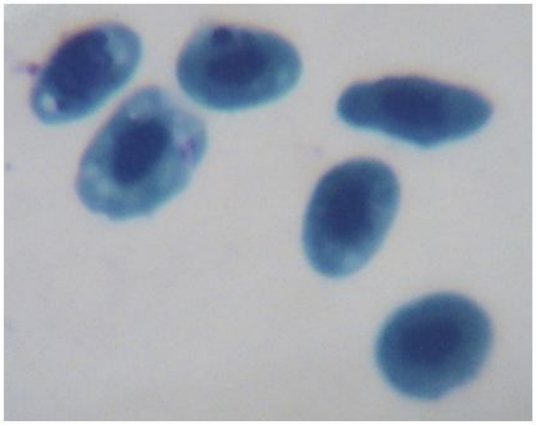


Fig 8. Micronuclei present in the erythrocytes

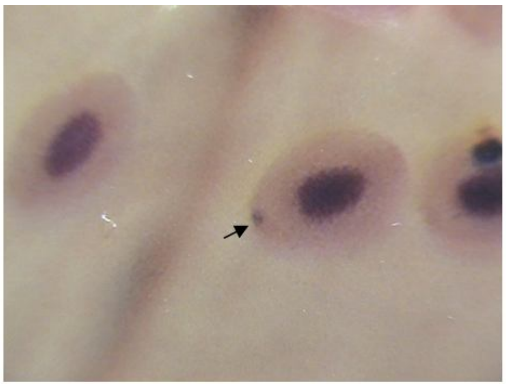


Fig .9. Micronuclei present in the erythrocytes

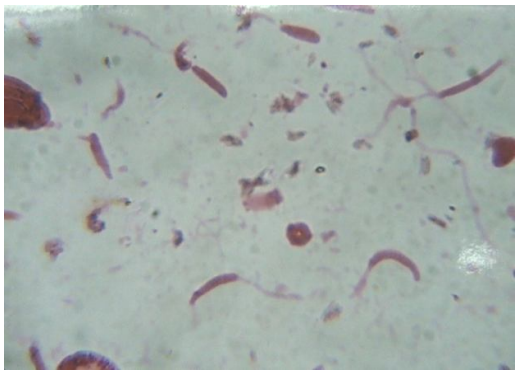


Fig. 10. Sickle shaped sperm

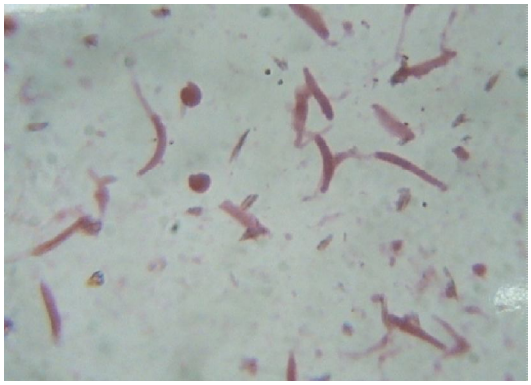


Fig 11. a. Sickle shaped sperm b. Normal sperm and c. Hook shaped sperm

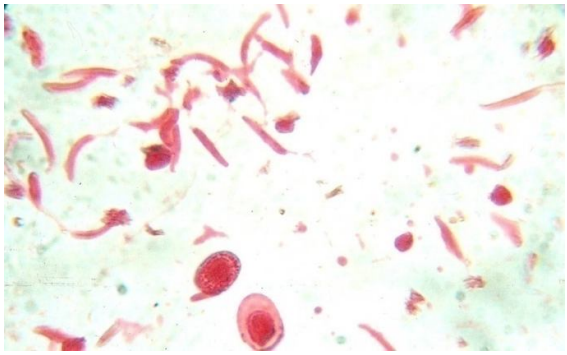


Fig. 12. 1. Bulged and 2 Sickle shaped sperms

Table 1. Frequency of chromosomal aberrations in *L. keralensis*, treated with different doses of carbaryl at different time intervals

Treatment	Control	G_I	G_{II}	G_{III}	G_{IV}
24h. M±SE	22±1	25±1	29±1	30±2	33±1
48h. M±SE	22±1	27±1	31±1	32±1	35.5±0.5
72h. M±SE	22±1	30.5±0.5	33±1	35.5±1.5	37.5±0.5*
96h. M±SE	22±1	28.5±0.5	32±1	34.5±1.5	36±1*
Average	22±1	27.75±0.75	31.25±1.0	33±1.5	35.5±0.75

* Significant compared to control (P<0.05)

Table 2. Percentage frequency of micronucleus found in erythrocytes of *L. keralensis*, treated with carbaryl .

Treatment	Control	G_I	G_{II}	G_{III}	G_{IV}
24h. M±SE	0.07±0.005	0.12±0.01	0.10±0.02	0.115±0.017	0.11±0.085
48h. M±SE	0.07±0.005	0.13±0.02	0.14±0.023	0.15±0.015	0.16±0.05
72h. M±SE	0.07±0.005	0.14±0.02	0.19±0.017	0.22±0.017	0.26±0.009*
96h. M±SE	0.07±0.005	0.14±0.02	0.20±0.02	0.24±0.014	0.25±0.005*
Average	0.07±0.005	0.132±0.28	0.15±0.08	0.18±0.015	0.19±0.036

* Significant compared to control (P<0.05)

Table 3. Percentage frequency of sperm abnormalities observed in *L. keralensis*, treated with different doses of carbaryl at different time intervals

Treatment	Control	G_I	G_{II}	G_{III}	G_{IV}
24h. M±SE	3.12±0.165	2.82±0.07	3.2±0.11	3.62±0.13	3.46±0.24
48h. M±SE	3.12±0.165	3.1±0.17	3.57±0.10	3.13±0.16	3.40±0.02
72h. M±SE	3.12±0.165	3.91±0.165	3.53±0.37	3.81±0.147	4.09±0.08*
96h. M±SE	3.12±0.165	3.45±0.04	3.5±0.13	3.33±0.165	3.40±0.026*
Average	3.12±0.165	2.63±0.44	3.45±0.17	3.47±0.15	3.58±0.09

Micronucleus

Results of present study showed significantly increased frequency of micronucleus on pesticide exposure when compared to control (Table 2). Further, the frequency of micronucleus increased with the increase in dose of carbaryl at increased time intervals. Highest frequency of micronuclei was obtained at higher dose (LD_{50}=100 and 75mg/kg b.w.) at 96 h (Fig ,8, 9 and 14).

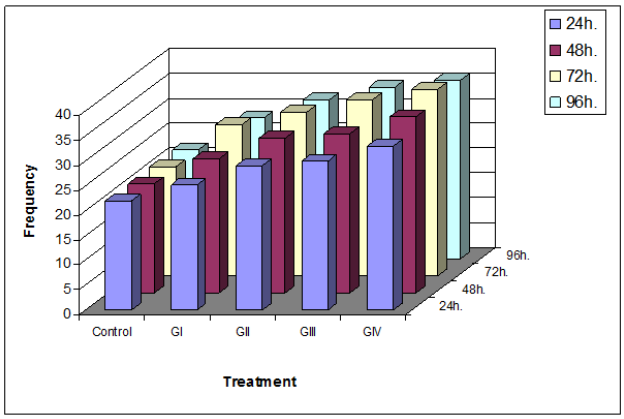


Fig.1. Frequency of chromosomal aberrations in *L. keralensis*, treated with carbaryl

Sperm abnormalities

The sperm abnormalities were observed in a wide variation in different treatment groups at different time intervals. But, carbaryl exposure for 96 h showed consistently significant values indicating carbaryl can induce sperm abnormalities at prolonged treatments. The inconsistency in the values of other groups may be due to wide individual variations. The results of sperm morphology assay indicate that carbaryl is effective in inducing sperm abnormalities at significantly higher levels of pesticide exposure at 96 h time intervals in lethal and sub lethal dose treatment schedules when compared to control (Table 3) (Fig 10, 11abc, 12 a b, and 15). The increase in sperm abnormalities was on time and dose dependent manner. There was no significant change in 72 h time intervals even at higher doses but was significant in higher doses at 96 h when compared to control.

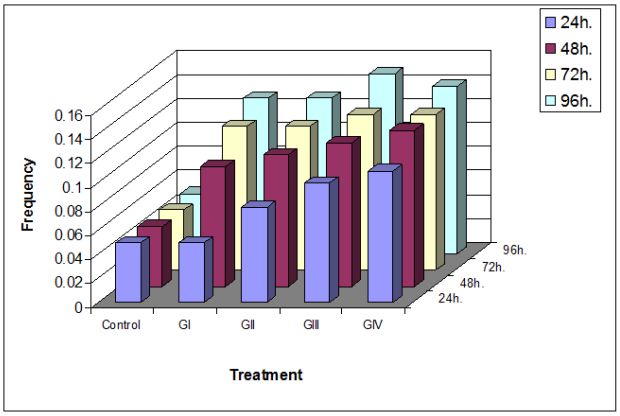


Fig. 2. Frequency of micronucleus found in erythrocytes of *L. keralensis*, treated with of carbaryl

DISCUSSION

Chromosomal aberrations

Chromosomal aberration test is a preliminary observation for evaluating the effect of environmental agents on the genetic material and problem of mutagenicity in exposed animals (Cohen and Hirschhoron 1971). In Western Ghats, many of the amphibians in agro ecosystem threatened due to pesticide effects. Due to the influence of pesticide in vertebrate including amphibians there is stimulus for invoking damage genetic material cause genetic defects and ultimately decline the amphibians. The radiation and bleomycin alkylating agents are high energy photons and affect the chromosome at G_1 phase. This will lead to double strand chromosome breakage or isochromatid breaks. This results in high percentage of chromosomal aberrations. The other organophosphate, organochlorine and carbamates they are less energetic because they affect only G_2 and S phase (Preston *et al*.1981). The toxicity causes effects on S and G_2 phase called S dependent. This will cause chromatid type breakages and deletions. The toxicity affects at G1 phase is called 'S' independent, this will cause chromosome type breakages (Preston *et al* 1981).

Carbaryl is also'S' dependent pesticides, so it has caused chromatid type of aberrations in *L. keralensis*. Genotoxicity effects due to carbaryl have not been studied extensively. However, It has been reported to be non-mutagenic in *E. coli* (Ashwood *et al*. 1972), *Bacillus Subtilis* (Shirasu *et al*.1976), *S. typhimurium* strains (Marshall *et al*. 1976) and in stationary phase cells of yeast, *S. cerevisiae* D_4 strain (Zimmermann *et al.*, 1984). In a newt, carbaryl exposure has caused broken DNA in red blood cells and has increased the frequency of lethal mutations in fruit flies (Siboulet *et al.* 1984). Carbaryl caused abnormal mitosis in Chinese hamster cells (Kozumbo, 1992) and breakages of chromosomes (Cox 1994). Our observation also similar to earlier workers, Ishidate and Odashima (1977) observed a strong positive response for carbaryl (30µg/ml) in chromosomal aberration assay using a Chinese hamster fibroblast cell line. Carbaryl induced predominanatly chromatid type's gaps, breaks, translocations, rings and fragmentations at after 48h treatment.

Carbaryl was negative in inducing chromosomal aberrations in Chinese hamster ovary cells without metabolic activation conditions but positive under metabolic activation condition (Muruli 1989). On daily oral doses of technical carbaryl (10mg/kg) suspended in pea nut oil to male albino rat, for a period of 5 days, there was a significant increase of chromosomal changes in the bone marrow cells of exposed animals was observed (Dikshith 1991). Carbaryl was mutagenic in gene mutation assay with cultured Chinese hamster V_{79} cells and inhibited mitosis spindle fiber formation in cultured in human embryonic fibroblast (IARC 1987). Supportive to our present findings, carbaryl exposure caused chromosomal aberrations significantly in Chinese hamster cells (Onfelt and Klasterska 1984). Mac Enaney (1993) reported that carbaryl (30mg/kgb.w.) induced chromosomal aberrations not significantly in rat at 48 h. A significant decrease in mitotic division was evident at higher doses in all durations against control cells, which indicates mitotoxic property of carbaryl. It was reported that carbaryl depresses mitotic activity of the cells in allium root meristems (Sneizko *et al*.1997; Nagpal *et al*.1998). Carbaryl induced genotoxic effects have been

reported by *in vitro* studies as mitotic aberrations in V_{79} Chinese hamster fibroblasts (Renglin 1999). Delescluse *et al*. (2001) have also reported that carbaryl provoked a strong DNA damaging activity in human lymphoblastoid cell lines. Carbaryl induced DNA damage and chromosomal aberrationsin human cell lines significantly in workers, who were exposed to carbaryl (Xia *et al*. 2005). It was reported that there was a significant decrease in mitotic index at higher doses against control (Verma and Bharadwaj 2003) indicates mitotoxic property of carbaryl. In another study, carbaryl caused a significantly higher frequency of chromosomal aberrations in kidney cells of fish *Channa punctatus*, indicates that carbaryl is a potent inducer of chromosomal aberrations (Pradipta Kumar Sarangi 2000). 100 and 125mg/kg b.w. of carbaryl doses were significantly induced chromosomal aberrations in *L. limnocharis* at 72 and 96 h (Krishnappa and Venkateshwarlu 2007). In the present study, Chromosomal aberrations were gradually increased on time and dose dependent manner, Chromosomal aberrations were not significant at 24 to 48 h exposure even at higher doses but they were significant at 72 and 96 h in higher doses, hence carbaryl is said to be mutagenic agent

Micronucleus

Micronucleus test is the simple method to screen the genotoxicity of the pesticide. Micronuclei are supernumerary nuclei visible by light microscopy in the cytoplasm of haemopoetic tissues or sometimes even in actively dividing cells. Also known as Howell-Jolly Bodies in mammals (Schlegel *et al*. 1986), they are formed in dividing cells when a centric chromosomes fragments or whole chromosome lags behind during anaphase because of clastogenic or aneuploidic. A negative response was in higher dose of carbaryl (140mg/kg b.w. Orally, 2 times 24 and 30h sampling time) was reported by Usha Rani *et al.,*(1980) with an adequate protocol for the mouse bone marrow micronucleus test. No increase in the rate of micronucleated polychromatic erythrocytes in the bone marrow of mice was found during *in vivo* nitrosation of carbaryl after administration of maximum nitrite (100mg/kg b.w.) (Seiler, 1976).

Carbaryl significantly induced high frequency micronuclei in bone marrow cells and peripheral erythrocytes of chick following intraperitoneal injection at 24 h (Bhunya and Jena 1995). Male and female rats were fed with carbaryl orally, it did not induce any significant level of micronucleus (Marshal 1996). Carbaryl is also an effective inducer of micronuclei in peripheral erythrocytes of fish C*hanna punctatus* (Pradipta Kumar Sarangi 2000). Rats fed with carbaryl 50, 100 and 200mg/kg body weight orally, did not cause any significant increase in chromosomal aberration (Marshal, 1989). A negative clastogenic response for carbaryl in the range of 50-200mg/l was reported by Ma *et al*., 1984, who has used the plant Tradescantia for micronucleus test. 125 and 100mg/kg b.w. of carbaryl doses were significantly induced micronucleus in *L. limnocharis* at 72 and 96 hrs (Krishnappa and Venkateshwarlu 2007). In contrast, to that the results of the present study indicate that the carbaryl is an effective inducer of micronuclei. In the present study, micronucleus were gradually increased on pesticide exposure as time and dose dependent manner, they were not significant at 24 to 48 h exposure even at higher doses but they were significant at 72 and 96 h in higher doses , hence it is considered as mutagenic agent.

Sperm morphology assay

Sperm morphology assay has been routinely performed to diagnose testicular damage in humans and domestic animals. Though, it is generally agreed that large reductions in sperm number, motility, or large increases in sperm with abnormal shapes are associated with reduced fertility (Amelar 1966). Studies evaluating the genetic consequences or sperm changes by chemical induction focused mainly on understanding the genetic basis of induced sperm shape abnormalities in mice. A number of evidences suggest that induced changes in sperm morphology reflect genetic damage in the male germ cell. First, indicates that sperm shaping is polygenetically controlled by numerous autosomal and sex linked genes including sex linked genes, including T-locus alleles (Bennett 1975). Meeker *et al*. (2004) revealed that the relationship between carbaryl exposure and increased DNA damage in human sperm. Carbaryl induced sperm abnormalities positively in earthworm *Metaphire posthuma* (Gupta and Saxena 2003). Many reports suggest that sperm DNA damage was related to fertilization and pregnancy (Sun *et al*. 1997; Zini *et al*. 2001; Benchaib *et al*. 2003; Carrel *et al*. 2003a; Henkel *et al*. 2004). Sperm chromosome aberrations were also reported to be associated with infertility, pregnancy loss, spontaneous abortions and birth defects (Hassold and Jacobs 1984; Shah *et al*. 2003; Carrell *et al*. 2003b). Most early abortions have gross chromosomal anomalies in sperm (Warburton *et al*. 1990). During the pre-conception window damage to spermatosomal DNA can be transmitted to the zygote and may cause early embryo death (Hales and Rabaire 1996).

Chromosome abnormalities, the majority of which are paternally derived can lead to abnormal reproductive outcomes, in which the most common is early loss of the pregnancy, as well as genetic diseases in offspring of most conceptions over 50 percent chromosomal defects, including aneuploidies and structural aberrations (McFadden and Friedman 1997). Chromosomal aberrations such as aneuploidy account for approximately 50% of sporoidic fetal losses prior to 15 weeks (Byrne and Ward 1994; Abruzzo and Hassold 1995). The impact of chromosomal abnormalities is greatest during fetal life when they have their highest frequency and represent a major cause of fetal loss. Approximately, 50% of all spontaneous pregnancy loss and the loss of very high proportion of all human conceptions are due to chromosomal abnormalities (Dinesh *et al*. 2003). The gradual increase in the percentage of abnormal sperm heads in mice in carbaryl treated series compared to control might be attributed to the damage caused by the insecticide on the DNA material as was reported by Bruce and Heddle (1979). It has also been reported that, there was an increase in sperm abnormalities in male rats fed with carbaryl for 60 days (Pant *et al*. 1996). Pant *et al* (2000) reported that by histopathological observation in the seminiferous tubules i.e., vacuolation of sertoli cells, disturbed spermatogenesis in rats by the treatment of carbaryl. Carbaryl caused low sperm counts and abnormal sperm in male workers, those who were exposed to carbaryl (Caroline Cox 1993). When they were given a single dose (not precisely indicated) of 0.25ml of the carbaryl by ip injection; however, there were no morphological disturbances in spermatocytes (Venkat Reddy *et al*.1974. Carbaryl increased the proportion of

abnormal sperm in humans workers, those who were exposed and reduced sperm abnormality (ability to move) in rats (Shtenberg and Rybakova 1968). Carbaryl was negative in inducing sperm abnormality in humans (IARC 1987). A 1980, U.S. Environmental Protection Agency (EPA) review cited four studies (three in rats, one another rodent) showing decreases in sperm numbers, an increase in sperm abnormalities and decrease in sperm mobility caused by carbaryl exposure. Similarly, In the earlier study, 100 and 125mg/kg b.wt of carbaryl doses were significantly induced sperm abnormalities in *L. limnocharis* at 72 and 96 h (Krishnappa and Venkateshwarlu 2007). Similarly in our present study, sperm abnormalities were gradually increased on treated frogs time and dose dependent manner, they were not significant at 24 to 48 hrs even at higher doses but they were significant at 72 and 96 hrs in higher doses, hence it is said to be mutagenic.

Conclusion

Present study, reveals that the carbaryl induced chromosomal aberrations mironucleus and abnormal sperm morphology were more significant when compared to control in *Limnonectes kerealensis*. Hence carbaryl considered as mutagenic agent.

Acknowledgement

The Authors are grateful to the authorities of Kuvempu University for support and help for the completion of this work. Also thankful to the chairman department of Biotechnology, Kuvempu University for providing microphotographs and SC/ST cell for providing financial assistance in the form of University fellowship.

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