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Evaluation of DNA Damage *in vivo* by Comet Assay and Chromosomal Aberrations for Pyrethroid Insecticide and the Antimutagenic Role of Curcumin

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ABSTRACT

Background: Esfenvelerate a synthetic pyrethroid insecticide, is widely used in the home environment and in agriculture because of its high activity against a broad spectrum of insect pests and its low animal toxicity .O**bjective of this study** was to evaluate the genotoxicity of esfenvelerate and the possible protective role of curcumin against this genotoxicity. **Material and methods:**Forty male albino rats were divided into 8 groups of 5 rats each: G1 served as control and G2 served as positive control received (100mg/kg curcumin), G3,G4 and G5 were orally administrated with (1/20 LD50, 1/40 LD50 and 1/60 LD50 of esfenvelerate) respectively and the last three groups(G6,G7and G8) were received the same doses of pesticide plus 100mg /kg curcumin for 28 days daily. Animals were sacrificed and bone marrow samples were collected for chromosomal aberration assay test and liver samples were used for DNA damage detection by comet assay. **Results:**chromosome aberration assay revealed that all the tested doses induced chromosomal aberrations (CA) such as centromeric gaps, chromatid gaps, chromatid deletion, dicentric chromosome, and ring chromosome. The alkaline comet assay showed significantly increased tail moment, tail length and tailed DNA % in liver cells of animals treated with esfenvelerate alone compared to control group. On the other hand, oral curcumin significantly ameliorated the genotoxicity induced by esfenvelrat. All these results clarified the efficacy of curcumin in amelioration of chromosomal aberrations of structures as well as DNA damage which may result from its antioxidant properties.

Key words: pyrethroid insecticide, comet assay, chromosomal aberrations, curcumin

INTRODUCTION

Pesticides have become an increasingly serious source of chemical pollution of the environment due to their extensive usage in agriculture **.**[1] Pesticides of the pyrethroid class, such as esfenvelerate are widely used because of their short biodegradation period and their low tendency to accumulate in organisms**.**[2] Pyrethroids are a class of neurotoxic pesticides registered for agricultural and residential use in the United States. Use of pyrethroids has continuously increased during the last two decades.[3] Pyrethroid pesticides such as cypermethrin and Fenvalerate show high toxicity to a wide range of insects, including some pesticide resistant strains and low toxicity to mammals and birds but when administered at a high dose, pyrethroids produce evident neurotoxicity in mammals. However, the current information is not sufficient to adequately assess the risk posed by fenvalerate to non-target organisms, though some work has been done to assess its toxicity to non target species**.** [4]Fenvalerate [(RS)--cyano-3-phenoxybenzyl (RS)- 2-(4-chloro-phenyl)-3-methyl-butyrate, a third generation synthetic pyrethroid pesticide is active against a wide variety of pests. [5]Carbonell *et al.* [6] found a strong effect of fenvalerate on the mitotic spindle apparatus resulting in the occurrence of C-mitoses. Fenvalerate has been reported to inhibit intracellular communication measured as inhibited metabolic cooperation between V79 cells.

Most of pesticide had been tested for their genotoxicity and cytogenicity using different testing assays**.** [7] Biological monitoring provides a useful tool to estimate the genetic risk deriving from an integrated exposure to a complex mixture of chemicals. Studies available in scientific literature have essentially focused on cytogenetic end-points to evaluate the potential genotoxicity of pesticides in occupationally exposed populations. A positive association between occupational exposure to complex pesticide mixtures and the presence of chromosomal aberrations (CA), sister-chromatid exchanges (SCE) and micronuclei (MN) has been detected in them . Genetic damage associated with pesticides occurs in human populations subject to high exposure levels due to intensive use, misuse or failure of control measures. [8] The comet assay,

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which is also referred to as the single cell gel electrophoresis assay (SCG or SCGE assay), is a rapid and quantitative_technique by which visual evidence of DNA damage in eukaryotic cells may be measured. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis. This assay has gained widespread use in various areas including human biomonitoring, genotoxicology, ecological monitoring and as a tool for research into DNA damage or repair_in different cell types in response to a range of DNA-damaging agents**.** [9]

In recent years, attention has been focused on whether naturally occurring compounds can modify the effects of various mutagens and carcinogens. Curcumin (diferuloylmethane) is a yellow orange dye derived from the rhizomes of Curcuma longa turmeric, which is used as a spice and food-colouring agent. The reports from *in vivo* and *in vitro* studies indicate that curcumin possesses antioxidant and anticarcinogenic properties**.** [10]Curcumin was found to have protective effect in cisplatin-induced DNA damage in PC12 cells**.** [11]This present study was undertaken with the objective of evaluating possible *in vivo* genoprotective effects of curcumin on esfenvelrate -induced genotoxicity in rats.

Material and Methods:

Chemicals

pesticide Esfenvelerate (purity 5%) was obtained from Kafr EL Zayate Pesticides & Chemicals, and antioxidant material Curcumin (purity 98% total curcuminoid content was obtained from Alfa Aesar company) Tumeric rhizome

animals

Forty male Wistar rats were obtained from Animal Breeding House of the Research Institute of Ophthalmology ,Giza, Egypt. Rats were housed in clean plastic cages with free access to food (standard pellet diet) and tap water *ad libitum*, under standardized housing conditions(12 h light/dark cycle, the temperature was 23 ± 2∘C, and a minimum relative humidity of 44%) in the laboratory animal room, body weight range of 145–155 g before being used for this study.

Experimental Design

animals were caged in 8 groups(5 animanls for each group), they had oral administration of insecticide or curcumin by gastric tuber daily for 28 days. :Forty male albino rats were divided into 8 groups of 5 rats each: G1 served as control and G2 served as positive control received (100mg/kg curcumin), G3,G4 and G5 were orally administrated with (1/20 LD50, 1/40 LD50 and 1/60 LD50 of esfenvelerate) respectively and the last three groups(G6,G7and G8) were received the same doses of pesticide plus 100mg /kg curcumin.

Experimental procedure :

- **For chromosomal aberration**

Colchicine (4mg/ b.wt) was injected intraperitoneally 2h prior to sacrifice of animals in order to arrest mitosis .Rat were sacrificed by cervical dislocation . Bone marrow of both femurs was removed by flushing with 0.075 M potassium chloride and incubated at 37◦ C for 30 min . It was centrifuged and fixed in cold methanol / glacial acetic acid 3:1,after 2h of fixation , the cells were centrifuge and suspended in the same fixative for 24h . The fixed cells were dropped on chilled slides from a height of about 2-2.5 m the slides were dried , stained with 10% Giemsa stain and 30-50% metaphase plates from each of animals per dose were scored and was done according to standard method of Preston[12].

- **Alkaline single cell gel electophorsis (comet assay)**

0.5 g of crushed samples were added to 1 ml ice cold PBS . This suspension was stirred for 5 min and filtered . Cell suspension (100µl) was mixed with 600µl of low-melting (0.8% in PBS) . 100 µl of this mixture spread on pre-coated slides were immersed in lyses buffer (0.045 M TBE , pH 8.4 and contain 2.5 %SDS) for 15 min . The slides were placed in electrophoresis chamber containing the same TBE buffer , but devoid of SDS . The electrophoresis conditions were 2 V/cm for 2 min and 100 m A ,staining with ethidium bromide 20 µg/ ml at 4C . The observation were evaluated with fluorescence microscope (with excitation filter 420-490 nm (issue 510 nm) . The comets tails lengths were measured from the middle of the nucleus to the end of tail with 40 X increase for the count and measure the size of the comet .

We use a comet 5 image analysis software developed by kinetic imaging Ltd . (liver pool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA ***damaged*** in the cells by measuring the length of DNA migration and percentage of migrated DNA it was done according to the method of Singh *et al.*[13]

Statistical Analysis

The results were expressed as mean ± standard error of means . The data were statistically analyzed using (ANOVAONE WAY) the program statistical package for social sciences (SPSS) version 17. The data means were considered different at $p < 0.05$.

RESULTS

-For chromosomal aberration

Table (1) summarized the total number and percentage of frequency of chromosomal aberrations in rat bone marrow cells after treatment with esfenvalerate alone or combined with curcumin.

The results showed an increase in total number and frequency of chromosomal aberrations in rat bone marrow cells after treatment with 1/ 20 , 1/40 and 1/60 of esfenvalerate. When treated rats administered with the same doses of esfenvalerate plus curcumin ,there was a decrease in total number and frequency of chromosome .

The percentage of aberration cells were 4%,6%,45.5.5%,25.5%,11%,30%,16.5%and9.5 %inG1,G2,G3,G4,G5,G6,G7, and G8s respectively, which revealed that pesticide alone increased the percentage of aberration cells while curcumin cause decrease in the percentage of chromosomal aberration cells. This result may be due to the protective effect of curcumin .

Alkaline single cell gel electophorsis (comet assay)

Table (2) indicated that The comet assay revealed that esfenvelrat significantly induced DNA damage. By comparing the values of mean± SD of tail length of the comet assay of(1/20, 1/40and 1/60) reached to 4.05 ± 0.11, 3.74±0.049and 3.13±0.36respectively. Moreover, [tailed % and tail moment]of 1/20, 1/40and 1/60reached to[22.00±3.00 and 16.10 ±0 .93],[14.33±2.08 and 13.07±.33] and[8.66±1.15and8.26± 2.34]respectively. Compared to control rats where its tail length reached 0. 96 ±0.08 and (tailed % and tail moment) reached to 3.33±0.57and1.077±0.19 ($p<0.05$).

On the other hand , esfenvelerate plus curcumin the values were mean± SD of tail length of the comet assay of(1/20, 1/40and 1/60),it was reached to3.57±0.16, 3.14±0.08 and 2.26±0.13 respectively. Moreover,[tailed % and tail moment] of 1/20, 1/40and 1/60reaching to[16.66±1.52and12.16±1.18],[9.66±0.57 and12.16 ± 1.18] and[6.33± 1.15and 4.72±0.54) respectively compared to control rats($p<0.05$).

Our findings indicated that all doses of esfenvelerat and esfenvelerate plus curcumin were significantly increased DNA damage levels. Curcumin inhibited increment of DNA damage levels But not reaching to normal.

DISCUSSION

Fenvalerate is an extensively used pesticide to which thousands workers and farmers worldwide are exposed, predominantly inhalation and skin absorption. Moreover, occurring as a single xenobiotic contaminant in genotoxic effects and mechanisms in human**.** [14] Chromosome aberration, The most frequently used test for genetic damage is the classical CA analysis of peripheral blood lymphocytes (PBLs). Since the 1960s, CA in PBLs has been used in occupational health surveillance programmes to assess genotoxic risks.[15] In epidemiological studies, it has been shown that people with elevated frequencies of CA in their peripheral blood lymphocytes have a significantly elevated risk of developing cancer**.** [16] The evaluation of chromosome aberrations is a fully accepted method to reveal genotoxicity, as it is indicative of real genetic effects**.** [17] Fenvalerate, a type II synthetic pyrethroid, has been reported to inhibit cellular communication in V79cells enhance the development of altered hepatic foci (AHF) in rat liver , and may act as a tumor promoter. However, the literature on the genotoxic properties of fenvalerate is equivocal**.** [18]In the present study significant induction of clastogenic activity of esfenverlerate has been observed sub acute (28ds) treatment. Institoˊris *et al.* [19] investigated the genotoxic effects of permethrin(PM) by structural and numerical CA in bone marrow cells. They showed that PM increased the number of numerical CA. Fahmy and Abdalla[20]reported clastogenic potential of cyhalothrin (synthetic pyrethroids) in fish, mouse and wistar rat in red blood cells and bone marrow respectively. *Xia et al.*[14]suggested that fenvalerate is one of the important genotoxic agents with a potential genotoxicity to human sperm. lambda-cyhalothrin has greater potential for inducing chromosomal aberration in long

duration treatments, while its effect has been found to be non-significant in short term treatment**.** [21] Chemicals that cause damage to lysosomes and membranes of cellular system, induce the release of lysosomal or other DNAase into the strand break and in those cells that survive sub lethal damage, such double strand breaks could have a variety of genotoxic effects such as mutation and chromosome aberrations**.** [22] Cypermethrin has been caused major degenerative changes in rat bone marrow cells**.** [23] The production of CA is a complex cellular process. The mechanism (s) of chromosome breakage and rejoining are not yet completely understood According to the prevailing theories, structural CA result from: (i) direct DNA breakage, (ii) replication on a damaged DNA template, (iii) inhibition of DNA synthesis,and other mechanisms such as topoisomerase II inhibition **.** [24] Dayal *et al.*[25] suggested that the tumour-initiating property of cypermethrin observed in their investigation may be attributed to several factors: first, its ability to interact with DNA and damage its structure. Such interactions are critical for the initiation of cells to transform into neo-plastic cells. A second possible mechanism is the induction of microsomal enzymes. Covalent binding of some pyrethroids to hepatic microsomal proteins has suggested the involvement of cytochrome P450-dependent mono-oxygenases in the metabolism of these pesticides. Giri *et al.*[26] indicated possible interaction between fenvalerate and DNA. The exact mechanism of interaction of fenvalerate with DNA is not known. The major route of metabolism of pyrethroids *in vivo* is via ester cleavage by esterase and oxidase attack, as well as hydroxylation of the terminal aromatic ring .The cleavage of the ester linkage in fenvalerate yielding two metabolites(4-chloro-alpha-1-methyl benzene acetic acid and 3-phenoxy benzoic acid)which considered to be the primary step in the biodegradation of fenvalerate in organs. None of these metabolites is reported to be mutagenic. Abdel-Aziz and Abdel-Rahem [27] observed that increasing dose of pyrethroid insecticide significantly increased the chromosomal aberrations and sister chromatid exchanges in bone marrow cells of Swiss albino mice. The highest concentration of Cyren (aorganophosphorus+ pyrethroid) was most effective to cause all types of chromosomal aberrations; this may be due to the elevation of cells sensitivity to higher concentration because high dose of mutagens causes rapid accumulation of mutated genes within the cells. Al-Attar[28] observed similar results when he used the insecticides that significantly increased the frequency of chromosomal aberrations in human blood lymphocytes. Turkez *et al.*[29] indicated that PM induced genotoxic and oxidative damage in rats. Our results found high frequency of chromosomal aberrations in fenvelerat alone or plus curcumin in all doses **.** Assayed *et al.*[30] indicated a significant induction of micronucleated polychromatic erythrocytes and a significant increase in the frequency of structural chromosomal aberrations – comprising chromatid-type and chromosome-type aberrations – in the bone-marrow of rats exposed to both single and repeated doses of cypermethrin, which is an indicative of potential clastogenicity. Nucleic acids system used as a marker for assessing the chromosomal aberration produced by the toxicity of xenobiotic such as pyrethroid pesticides. The significant decrease of DNA and RNA content in liver suggested the increase in chromosomal aberration. [31]Abu-Aita and Yassa[32] recorded chromosomal aberrations were in the form of structural and numerical aberrations. The structural aberrations included gap, break, deletion, fragment, ring, centromeric attenuation and pu-lverization when treated rats with deltamethrin at a dose of 0.26mg / kg b. wt. (about 1/100 of the LD50) orally by stomach tube twice weekly for 8 successive days. *Ila et al.*[33] reported that -cyfluthrin treatment increased the frequency of SCAs in rats, and cultured human peripheral blood lymphocytes. The potential genotoxicity of a commercial formulation of a-cypermethrin on human peripheral lymphocytes was examined *in vitro* by sister chromatid exchange (SCE), chromosomal aberrations (CAs), and micronucleus (MN) tests. cypermethrin significantly induced SCEs MN, and CAs and decreased the proliferation index and reduced both the mitotic index and nuclear division index. [34]Mitoic index decreased significant in both of low dose and high dose after 14 d treated

by Cyfluthrin which indicated inhibition of cell proliferation in bone marrow subsequently the frequency of aberrant cells.[35]

In fact, curcumin was reported to have free oxygen radicals and lipoperoxyradicals scavenging capacity and, anti-clastogenic activity due to its polyphenolic contents.

Various chemical compounds can induce base alterations in DNA. Chemicals can bind to DNA forming bulky adducts. Alkylating agents can add alkyl groups to DNA inducing secondary variations such as base loss leading to an a purinic or a pyrimidinic site (AP site) and to other damages such as DNA–DNA or DNA–protein crosslinks. [36] These DNA alterations can be transformed to DNA strand breaks (single and double). These last types of DNA damage can be produced both endogenously or by different types of exogenous chemicals and environmental agents. [37] Alkaline comet assay for the detection of DNA damage mainly reveals single or double DNA strand breaks and alkali-labile sites and has been successfully used for biomonitoring in populations exposed to various xenobiotics. [38] The single-cell gel electrophoresis (comet assay) is the simple stand most sensitive method that can measure and identify DNA damage at the cellular level. [39] Any damage to the DNA in the form of strand breaks leads to changes in the cell integrity, which in turn can lead to abnormal cellular activity leading to toxicity and ultimately cell death.[40,41] This may be partially due to the generation of oxidative lesions which reflects oxidized purine and pyrimidinic. Pesticides may induce oxidative stress, leading to the generation of free radicals and causing lipid peroxidation, and maybe cause molecular mechanism that gives rise to pesticide induced toxicity. [42] Excessive production of oxidants can result in oxidative damage, due to the oxidation of lipids, proteins and DNA . There is increasing evidence that oxidative stress, particularly stress caused by reactive oxygen species and reactive nitrogen species, can lead to numerous inflammatory degenerative diseases. [43] The practice of the single cell electrophoresis test (comet assay) has obtained an increasing acceptance in the genetic toxicology field. [44]Some of its advantages are: damage detection at an individual cell level, high sensitivity (mainly under alkaline conditions), as well as the possibility to use enzymes or antibodies to detect specific types of damage.[45] Cypermethrin was found to induced single strand breaks in the DNA as assessed by comet assay and caused increase in the comet tail length with increase in pesticide concentration. [46] The molecular mechanisms of the genotoxicity of cypermethrin (synathic pyrethroid) not yet elucidated and require further studies. Due to the hydrophobic nature and small molecular size, cypermethrin passes through the cell membrane and reaches the nucleus. It is suggested that within the nucleus, cypermethrin binds to DNA through the reactive groups of its acid moiety, leading to destabilization as well as unwinding of the DNA, which could be a possible mechanism for its genotoxicity. [47] 60 days for PERM treatment induced DNA damage in striatum of rats. This damage was related to an oxidative origin because the simultaneous antioxidant *in vivo* treatment with vitamin E alone significantly reduced the % of Tail DNA values. [48] DNA damage was observed in lymphocytes of workers occupationally exposed to pesticides such as cypermethrin. [49]Our findings agree with Sankar *et al* . [50]who showed that curcumin caused reduction in DNA damge after treated by cypermethrin and Mixture pesticides caused DNA damge was revealed by tail DNA % (TD%) and tail moment (TM) . Issam *et al.*[51] found significantly higher DNA damge ($P \leq 0.05$) after the administration of deltamethrin at a low dose (0.003 mg/kg bw/d)in whole blood cells by comt assay. put ref after fullstop

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Table 1. Effect of fenvalerate alone and fenvalerate plus curcumin on chromosomal aberrations in rat bone marrow cells.

Treatment		Total no. examined cells	Total no.of normal cells	Total no.of aberr cells	Structure aberration							Nuerical aberri polypoid
					g	b	td	r	f	d	min	
G1	no	200	192	8	3	4	0	1	0	0	0	0
	%	100	96%	4%	1.5	2	0	0.5	0	0	0	0
G2	no	200	188	12	5	4	1	1	1	0	0	0
	%	100	94%	6%	2.5	2	0.5	0.5	0.5	0	0	0
G3	no	200	109	91	40	24	3	4	12	4	4	8
	%	100	54.5%	45.5%	20	12	1.5	2	6	2	2	4
G4	no	200	149	51	20	16	0	3	4	0	8	0
	%	100	74%	25.5%	10	8	0	1.5	2	0	4	0
G5	no	200	178	22	12	8	0	2	0	0	0	0
	%	100	89%	11%	6	4	0	1	0	0	0	0
G6	no	200	140	60	30	19	0	4	1	0	0	6
	%	100	70%	30%	15	9.5	0	2	0.5	0	0	3
G7	no	200	167	33	18	10	0	2	0	0	0	3
	%	100	83.5%	16.5%	9	5	0	1	0	0	0	1.5
G8	no	200	181	19	8	10	0	1	0	0	0	0
	%	100	90%	9.5%	4	5	0	0.5	0	0	0	0

(g)chromited gap(td) chromited deletion (r) ring (f) fragment (b)chromited break (d) dicentric ((min) minute

Table (2) Commet assay of liver for control, esfenvlerate and esfenvlaret plus curcumin treated group.

TREATMENT	Tail length µm	Tail DNA %	Tail moment unit
G1 negative control	0.9667 ±.08505	3.3333 ±0.57735	1.07767±.195121
G2 positivecontrol	1.0800 ±.03000	3.0000 ±1.00000	1.14900 ± .153245
G3 1/20 LD50 of esfenvelerate	4.0567 ±0.11015a	22.0000±3.00000a	16.10033 ±0 .934958a,
G4 1/40 LD50 of esfenvelerate	3.7467 ±0.04933a	14.3333±2.08167a	13.07767±.334264a
G5 1/60 LD50 of esfenvelerate	3.1367 ±0.36019a	8.6667 ±1.15470a	8.26467± 2.349334a
G6 1/20 LD50 of esfenvelerate+curcumin	3.5700 ±0.16823b,c	16.6667±1.52753b,c	12.16867±1.186232b,c
G7/40 LD50 of esfenvelerate+curcumin	3.1467 ±0.8737b,c	9.6667±.57735 b,c	9.48967±.664407b,c
G8 1/60 LD50 of esfenvelerate+curcumin	2.2633 ±0.13051b,c	6.3333 ± 1.15470b,c	4.72467±.541791b,c

All data are expressed as means±SE; a significant different between esfenverate treatment alone and control($p<0.05$). b significant different between esfenverate treatment with curcumin and control($p<0.05$).c significant different between esfenverate treatment alone and esfenverate treatment with curcumin($p<0.05$).

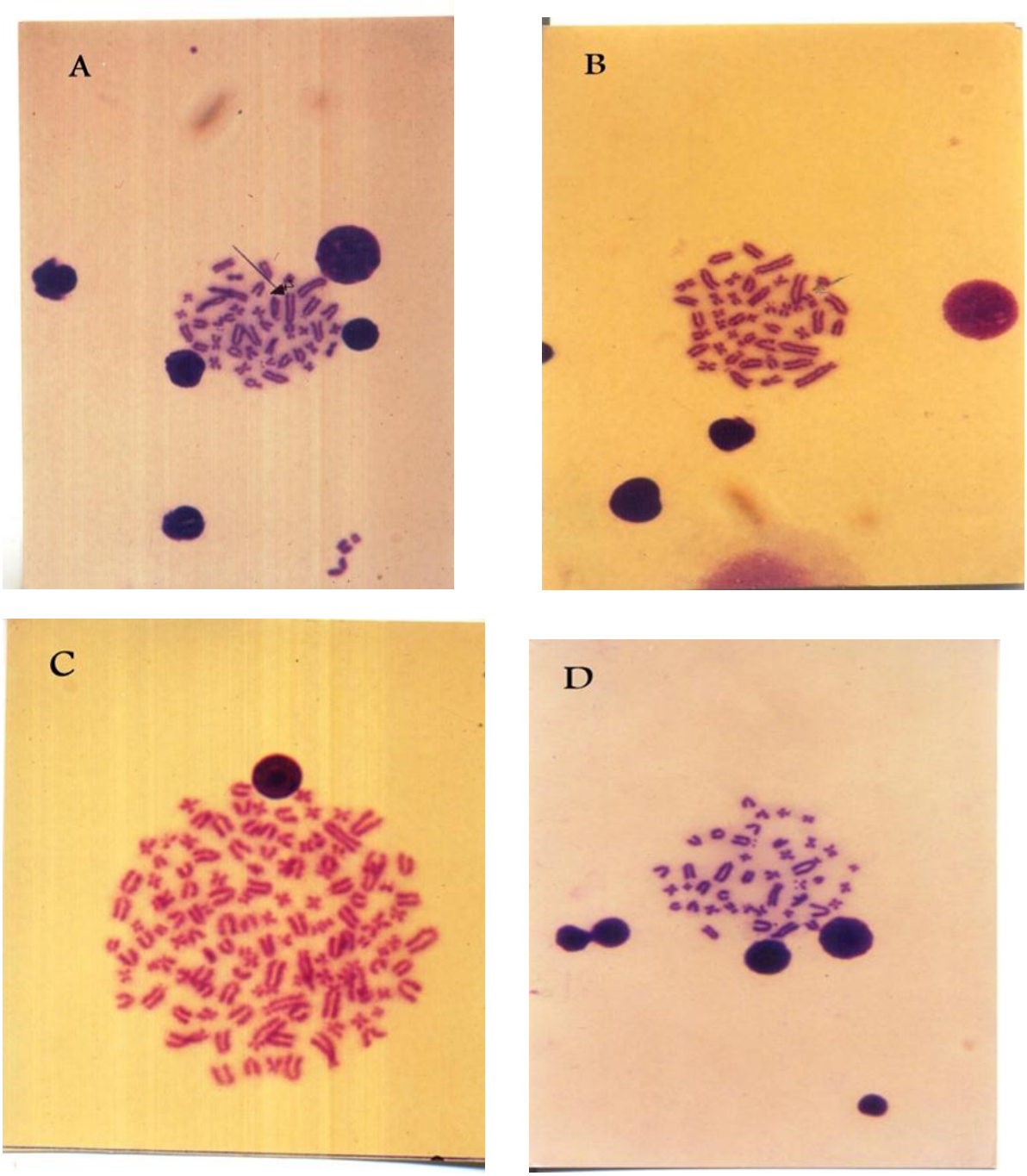

Fig (1): some of chromosomal aberrations induced by esfenvlarate in rat bone marrow cells. (A) Dentric(B) Break (c) Poly ploid (D) Ring

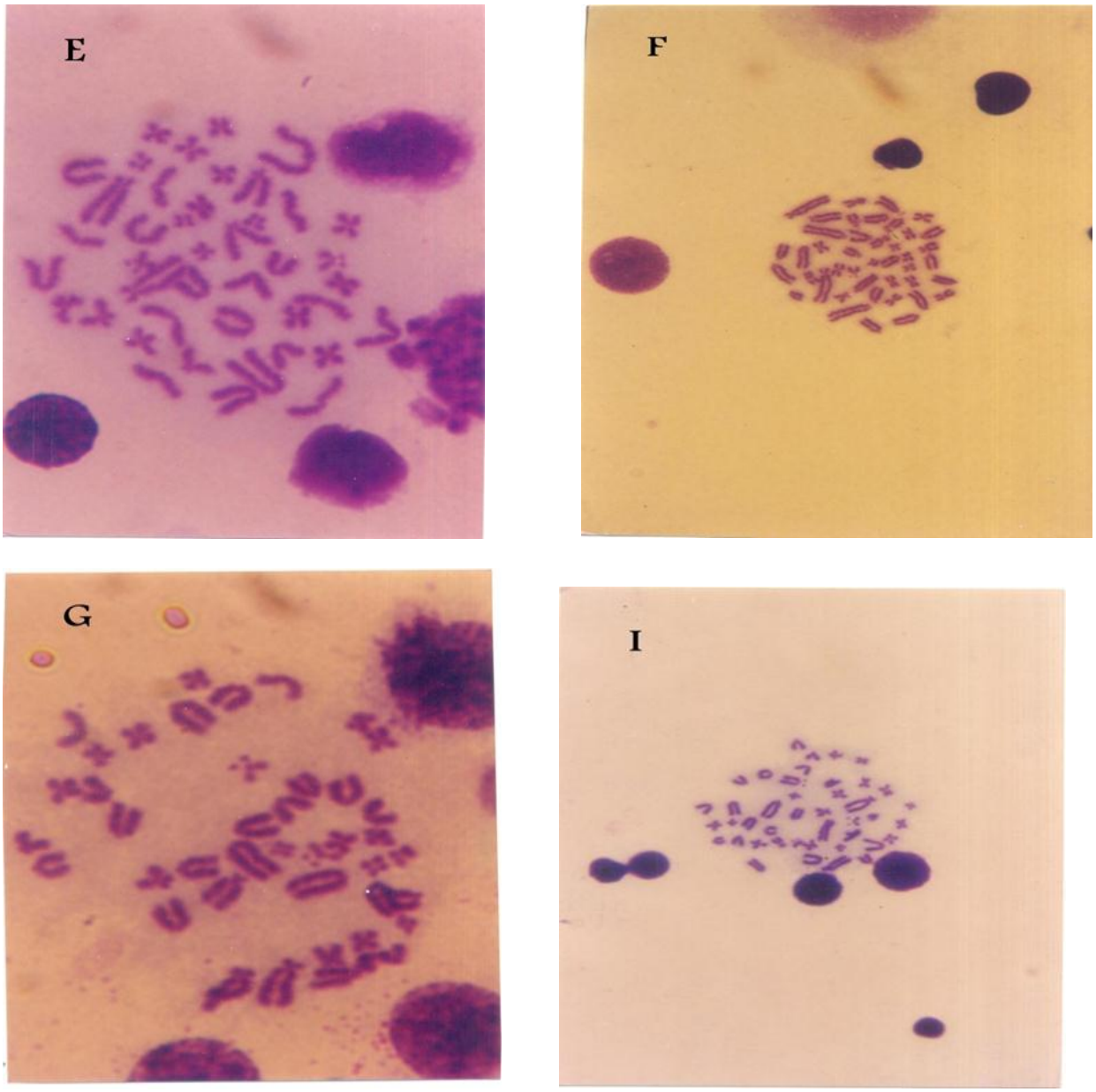

Fig (2): some of chromosomal induced by esfenvlarate in rat bone marrow cells.
(E) Normal (F) gap (G and I) minute