

GENOTOXICITY OF PATULIN IN BONE MARROW CELLS OF NEWLY BORN RATS

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Patulin is a kind of mycotoxins generated from many species of *Aspergillus*, *Byssochlamys* and *Penicillium*. Patulin is a contaminant of mature fruits (as apples) and vegetables used by human populations. Patulin has been scored to have carcinogenic, teratogenic and mutagenic properties (Alves *et al.*, 2000).

A mycotoxin is a harmful secondary metabolite which synthesized by many fungus species, it has the ability to cause diseases and death in animals and humans. Treatment of animals with patulin induces several biochemical and histopathological changes in organs such as kidneys, liver, thyroid, thymus,, endocrine system, gastrointestinal system, reproductive organs and brain (Bennett and Klich, 2003; Turner *et al.*, 2009; Srinivasan *et al.*, 2019).

MATERIALS AND METHODS

Patulin was purchased from Sigma-Aldrich (St. louis MO, USA). It was dissolved in dist. water, patulin was orally injected for 35 successive days to the experimental animals with a dose of 0.002 mg/kg.b.w which equivalent to 1/60 of

LD₅₀ (50 mg/kg b.w.) according to the literature of McKinley *et al.* (1982).

In the present study, twenty four white albino rats (*Rattus norvegicus*) were used. Rats were of both sexes (males and females) of about 3 months old. Their body weight ranged between 140±10 gm. The animals were purchased from House of Animal in Faculty of Veterinary Medicine (Zagazig Univ.). The rats were protected under hygienic condition, water *ad libitum* and fed on a balanced ration, bedded with wood shavings and housed in metal cages. Rats were randomly classified into two main groups; each group included twelve rats (3 mature males and 9 mature virgin females) as follows:

- The control group; animals were orally injected with dist. water for 35 successive days using a metallic stomach tube, then each three females were caged with one male for mating.
- The treated males and females group (Patulin Treated Group), both males and females were orally injected by patulin (0.002 mg/kg b.w.) for 35 successive days using a metallic gastric tube then, each three of treated females were caged with one treated male for mating.

Techniques of investigation, the smears of vagina were taken and examined daily. Mating was confirmed by the existence of either a vaginal plug (was rarely scored in some cases) or of sperms in the taken smear or of both. The day of this discovery was named 'Day 0' of pregnancy. The pregnancy continued normally 21-22 days (Huggett and Pritchard 1945).

After 14 days from parturition, fourty of the newly born rats (two or three babies from each mother) were randomly used for the examination of chromosomal abnormalities in the cells of bone marrow.

Reagents used in the present work were carefully prepared as follows:

- Colchicine solution (0.04%): 40mg of colchicine powder was dissolved in 100 ml of dist. H₂O then, kept in freezer.
- Phosphate buffer saline (PBS): 0.2 gm KCl; 1.874 gm Na₂HPO₄; 0.2gm KH₂PO₄ and 8gm NaCl were completely dissolved in 1L of distilled water.
- Hypotonic solution (KCl): From KCl powder 5.6 gm was dissolved in one liter of dist. water. It was carefully warmed (37°C) before use directly.
- Clarke's fixative: was prepared freshly as methyl alcohol/glacial acetic acid (3/1 V/V).
- Giemsa stain (1.5%): It was usually prepared using Genest and Auger (1963) method.
- Metaphase chromosomes were obtained from bone marrow of two or three ran-

domly selected babies for each mother. Each newly born rat was intraperitoneally injected with 0.04% solution of colchicine (0.25 ml/70 gm b. wt.).

- Exactly 2 hrs after the injection of colchicine, the rats were sacrificed to obtain the femurs and cutting off their epiphyses. Injection of about 6 ml of PBS into each femur and collection of the cell suspension in a sterile tube (a tube for each femur) was carried out.

The cell suspension was directly centrifuged (10 min at 1000 rpm) the supernatant was removed and 6 ml of hypotonic solution was added to the cells and the suspension was incubated at 37°C for 20 min, then centrifuged again (for 10 min at 1000 rpm) and the supernatant was removed.

- Six ml of Clarke's fixative was added, the cells gently mixed and kept for 20 min in the freezer.
- The suspension was centrifuged for 10 min at 1000 rpm and the supernatant was removed (this step was repeated three times).
- Addition of 1 ml of the fixative to obtain a good cell suspension that was dropped on cold and clean glass slides and was allowed to dry in air and stained using Giemsa stain.

The metaphases were examined by using light microscope (X10 eye piece and X100 oil immersion objective lens). Fifty metaphases were randomly examined for

each rat and photographs were taken when necessary.

All data was expressed as a mean \pm standard error of means of the two groups of animals. It was analyzed using SPSS version (15.0). Statistical analysis was applied using one-way analysis of variance ANOVA test (F-test). Duncan's multiple range test was applied and its significant level was defined as ($P < 0.05$) according to Snedecor and Cochran (1982).

RESULTS AND DISCUSSION

It is well known that the diploid chromosome number of rat is 42 (Fig. 1). They include 40 autosomes and 2 sex chromosomes. The metaphase spreads were photographed karyotyped according to the system of Hungerford and Nowell (1963)

Patulin is a mycotoxin found in many kinds of food, especially apple fruit and its products (apple juice), pears, other fruit and fruit juices, baby food and mouldy bread (Paster *et al.*, 1995).

From the results of the present investigation, it was clear that patulin (0.002 mg/kg b.w. for 35 successive days) have a genotoxic effect on mitotically dividing cells. This was indicated by the significant increasing in the total chromosomal abnormalities (numerical & structural) in the bone marrow cells of the rats' babies which were taken from the first generation of patulin treated parents (Table 1 and Fig. A).

As for the numerical chromosomal aberration; the aneuploid metaphases, especially hypodiploid (those with reduced number of chromosomes) and polyploidy (metaphases contains more than two sets of chromosomes) were significantly recorded in the bone marrow cells of those babies at a frequency more than normal.

The total numerical aberrations (10.4%) were statistically significant compared with the control (1.6%). Where, polyploidy reached to 15 case compared with 6 cases in control group (Fig. 2).

Hypodiploid metaphases were recorded in 85 case compared with 7 cases in the control group. These metaphases appeared in the form of variable number of chromosomes for example, metaphases which contained 37 chromosomes (Fig. 3) and five of which were randomly analyzed in patulin treated group (Fig. 4 and Table 2).

By the analysis of several hypodiploid metaphases, it was clear that, all the different autosomal groups were affected by the patulin treatment in certain chromosomal pair. In group (1-3) the chromosomal pair number one was the most missing chromosomes, in group (4-10) the chromosomal pair number 10 was the most missing chromosomes, in group (11-13) the chromosomal pair number 11 was the most missing chromosomes. In group (14-20) the missing chromosome belonged to chromosome pair number 20. On the contrast, the sex chromosomes were not affected by the patulin treatment

and there was no missing chromosomes scored (Fig. 4).

These data were confirmed by those of Roll *et al.* (1990) who reported that mycotoxin like patulin cause numerical chromosomal aberrations. Also, Elhajouji *et al.* (1995) concluded that patulin and citrinin induce genotoxicity in the chromosomes. The numerical chromosomal aberrations (aneuploidy) induced by mycotoxins are thought to contribute clearly to animal and human carcinogenicity (Pfeiffer *et al.*, 1998).

By the analysis of several hypodiploid metaphases, it was clear that, all the different somatic autosomal groups were affected by the patulin treatment and this was supported by the absence of certain chromosomal pair. On the contrast, there was no missing of sex chromosomes scored.

In our view, It seemed to believe that the significant appearance of cells with more (polyploidy), or less (hypodiploid) number of chromosomes in bone marrow of rats' babies after patulin administration to both of their parents might have resulted from patulin genotoxicity inducing endo-reduplication or disturbances in mitotic spindle apparatus, this may leads to impairment of the disjoining process (nondisjunction) where the chromosomal pair go to only one cellular pole and none to the other. These toxic effects resulted from patulin treatment negatively affect the cell division and hence cell cycle.

This was more explicated by Glaser and Stopper (2012) who explained that, patulin treatment induced cross-linking in DNA, DNA mutations, induction of nucleoplasm bridges, micronuclei formation, cell cycle delaying, alteration in cell proliferation and centrosome amplification which have an important function in the figuration of mitotic spindles in Chinese hamster fibroblast (V79) cells.

In addition, Srinivasan *et al.* (2019) added that patulin damages many important organs such as liver and kidneys. It induces the production of reactive oxygen species within the cell. Patulin induces damage in DNA leads to cell cycle stopping. It also affects protein expression engaged in cell junctions, protein synthesis and gene expression. Patulin causes DNA damage and arresting cell cycle in the S phase and at the G0-G1 transition (Saxena *et al.*, 2009).

From the data of the present study, the structural chromosomal aberrations significantly increased and were more abundant than numerical aberrations. The dicentric chromosomes were the most abundant one. In addition, the exchange chromosomes, Deletion, ring chromosome and acentric fragments were also recorded.

The dicentric chromosomes was the most abundant aberration than the others (Fig. 5). Metaphases with dicentric chromosome reached to 68 occasions versus 3 occasions in control group. Additional forms of damage were clearly developed, these included exchange chromo-

somes that reached to 38 cases (Fig. 5). Deletion chromosomes also developed and reached to 35 cases (Fig. 5). Other aberrations were also observed such as ring chromosome that detected in 33 cases. Acentric fragments that scored in 14 case (Fig. 5).

The results of the present work were agreed with those of Zhou *et al.* (2009) who added that a patulin treatment clearly leads to micronuclei formation, multinucleate and binucleated cells, inducing damage in the chromosomes in hepatoma HepG2 cells of human.

Intraperitoneal injection of patulin induces the frequency of micronuclei in polychromatic erythrocytes and normochromatic erythrocytes, chromatid breaks, chromosomal aberrations and gaps in cells of bone marrow of male Kunming mice (Song *et al.*, 2014).

In our opinion, the significant increasing in chromosomal abnormalities (structural) in the cells of bone marrow of newly born rats after patulin treatment of their parents might be resulted from patulin induction to intracellular reactive oxygen species and decreasing the antioxidant defense system enhancing the oxidative stress on the vital components of the cell (chromosomes). This opinion will be the aim of a research in another future study.

But now, we can support our view generally, as confirmed by another authors such as Srinivasan *et al.* (2019) who explained that patulin reduces cellular anti-

oxidant level due to its ability to induce the formation of reactive oxygen species. This leads to deactivation or activation of many enzymes and proteins which play important role in cellular apoptosis and survival. Moreover, patulin causes oxidative stress in endoplasmic reticulum, mitochondria and DNA, subsequently, enhancing the cell cycle arrest.

Similar data were published by Barhoumi and Burgharli (1996) who recorded that patulin inhibits many enzymes as DNA and RNA polymerase and retard transcription process and translation. Patulin induce numerical chromosomal aberrations, significant increase in the frequency of sister chromatid exchange and oxidative stress to DNA in human cells (Liu *et al.*, 2003).

Liu *et al.* (2007) added that chromosome gaps and breaks were scored in Chinese hamster ovary cells exposed to patulin treatment. Patulin cause chromosomal aberrations and negatively affect chromatin function, cell division, mitosis and meiosis and DNA replication (Huda and Sami 2018).

In conclusion, from the results of the present investigation it was clear that, the patulin is a genotoxic agent; it induced a significant increase in both structural and numerical chromosomal aberrations in the bone marrow cells of the newly born rats which were taken from the first generation of patulin treated parents.

SUMMARY

Patulin is a toxin generated by many kinds of fungi as *Penicillium*. It is a known contaminant of ripe fruits especially ripe apples. Patulin may be mutagenic, cytotoxic, teratogenic and clastogenic agent. Thus, the present work was aimed to evaluate the possible genotoxic effect (chromosomal aberrations) that may be induced by patulin (0.002 mg/kg b. w.) in the bone marrow cells of the newly born rats after oral injection of patulin to both of their parents. The results concluded that, patulin elicited a significant increase in total chromosomal aberrations (numerical and structural) in the bone marrow cells of these rats babies.

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Table (1): Chromosomal aberrations frequencies in the bone marrow cells of babies which were taken from the first generation of untreated and patulin treated parents.

Groups	No of newly born rats	No of scored cells 50/young	Chromosomal aberrations								
			Numerical aberrations			Structural aberrations			Total aberrations		
			No	%	Mean± SE	No	%	Mean ± SE	No	%	Mean ± SE
Control group	20	1000	16	1.6	0.8 ± 0.186 ^b	14	1.4	0.7± 0.127 ^d	30	3.0	1.5± 0.235 ^b
Patulin treated group	20	1000	104	10.4	5.2± 0.450 ^a	192	19.2	9.6± 0.520 ^a	296	29.6	14.8± 0.247 ^a

Means within the same column in each category carrying different letters are significant at ($p \leq 0.05$) using Duncan's multiple range test, where the highest mean value has symbol (^a) and decreasing in value were assigned alphabetically.

Table (2): Percentage of missing chromosomes in the metaphases of the rats babies which were taken from the first generation of patulin treated parents.

	The chromosomal groups	The missing chromosome/s	
		From pair number	%
Metaphases with only 37 chromosomes	1-3	1, 3	26.0%
	4-10	9, 10	11.0%
	11-13	11, 12, 13	6.6%
	14-20	14, 15, 18, 20	5.7%
	Sex-chromosomes	-	0.0%

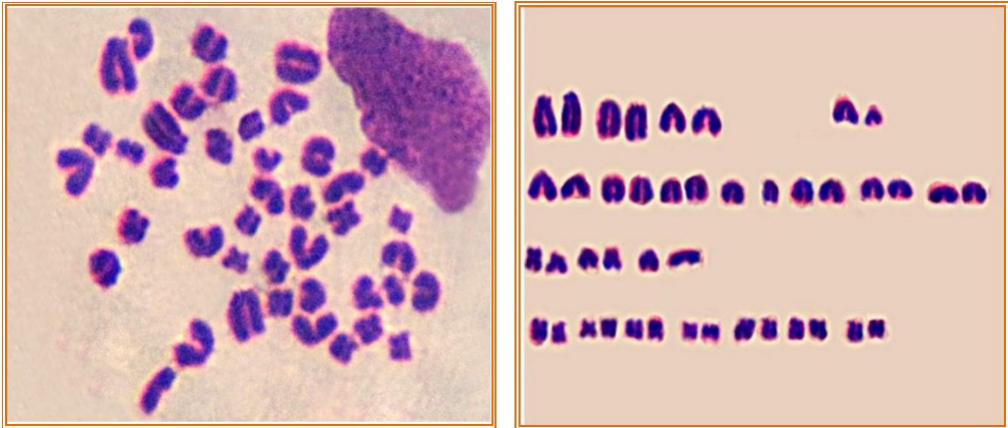


Fig. (1): Metaphase spread from bone marrow cells of newly born rats (on the left) and its karyotype (on the right) from control group.

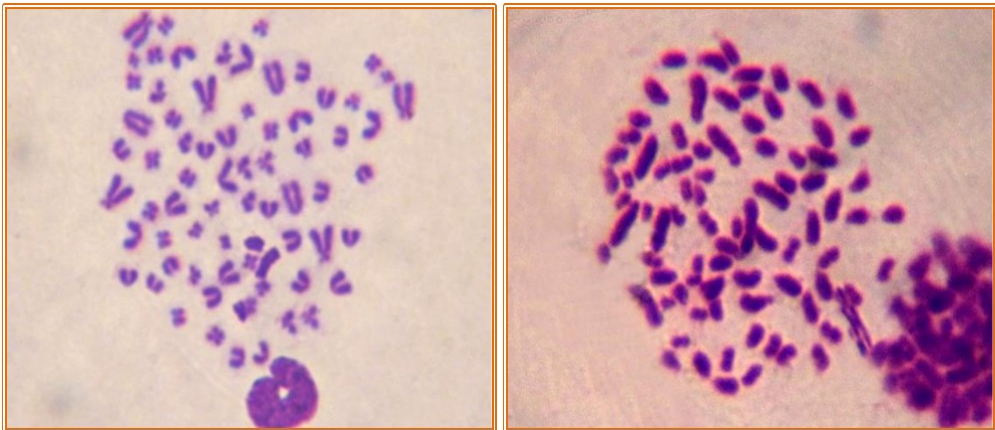


Fig. (2): Metaphases from cells of bone marrow of babies which were taken from the first generation of patulin treated parents showing polyploidy (on the left) and shrunken chromosomes (on the right)

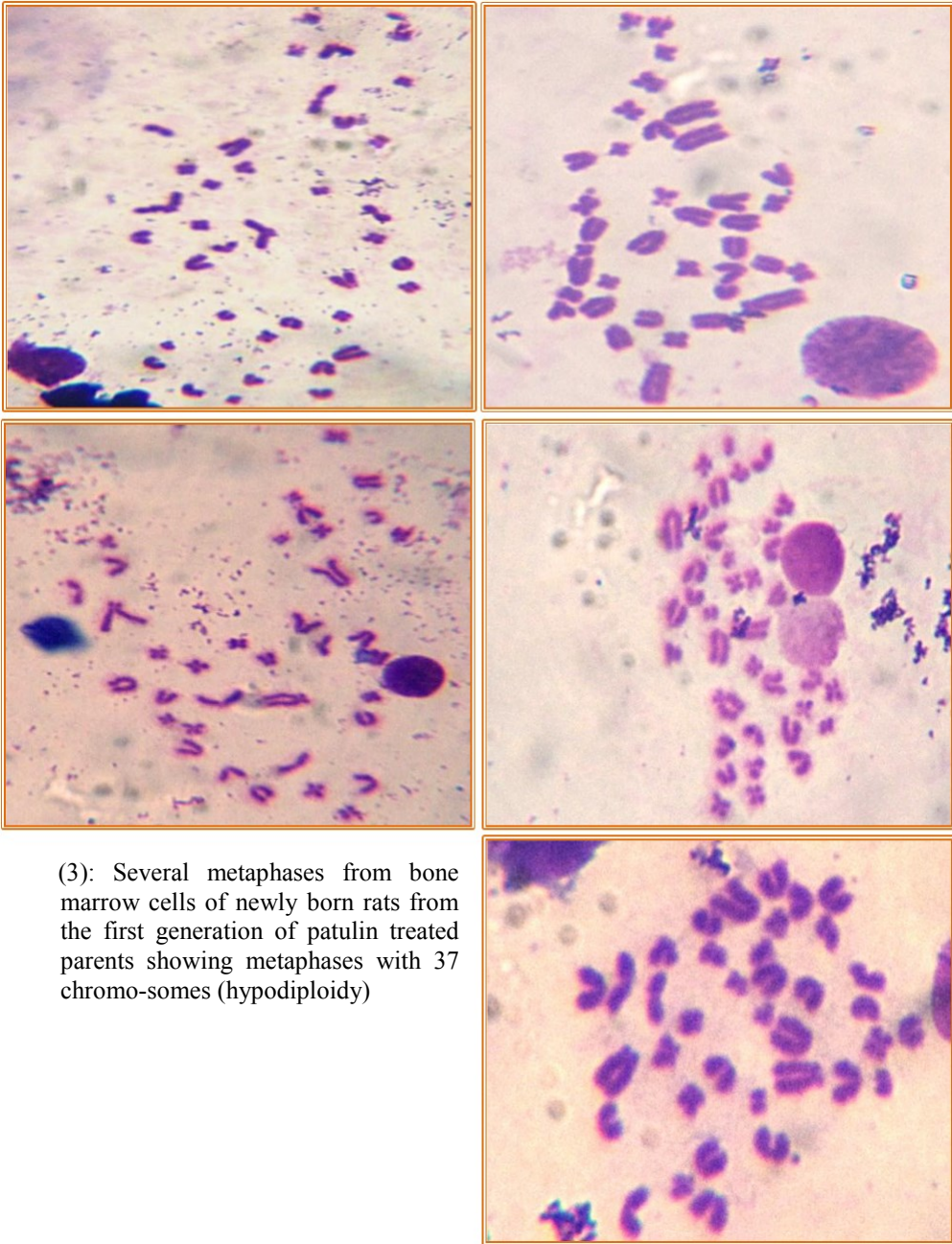


Fig. (3): Several metaphases from bone marrow cells of newly born rats from the first generation of patulin treated parents showing metaphases with 37 chromo-somes (hypodiploidy)

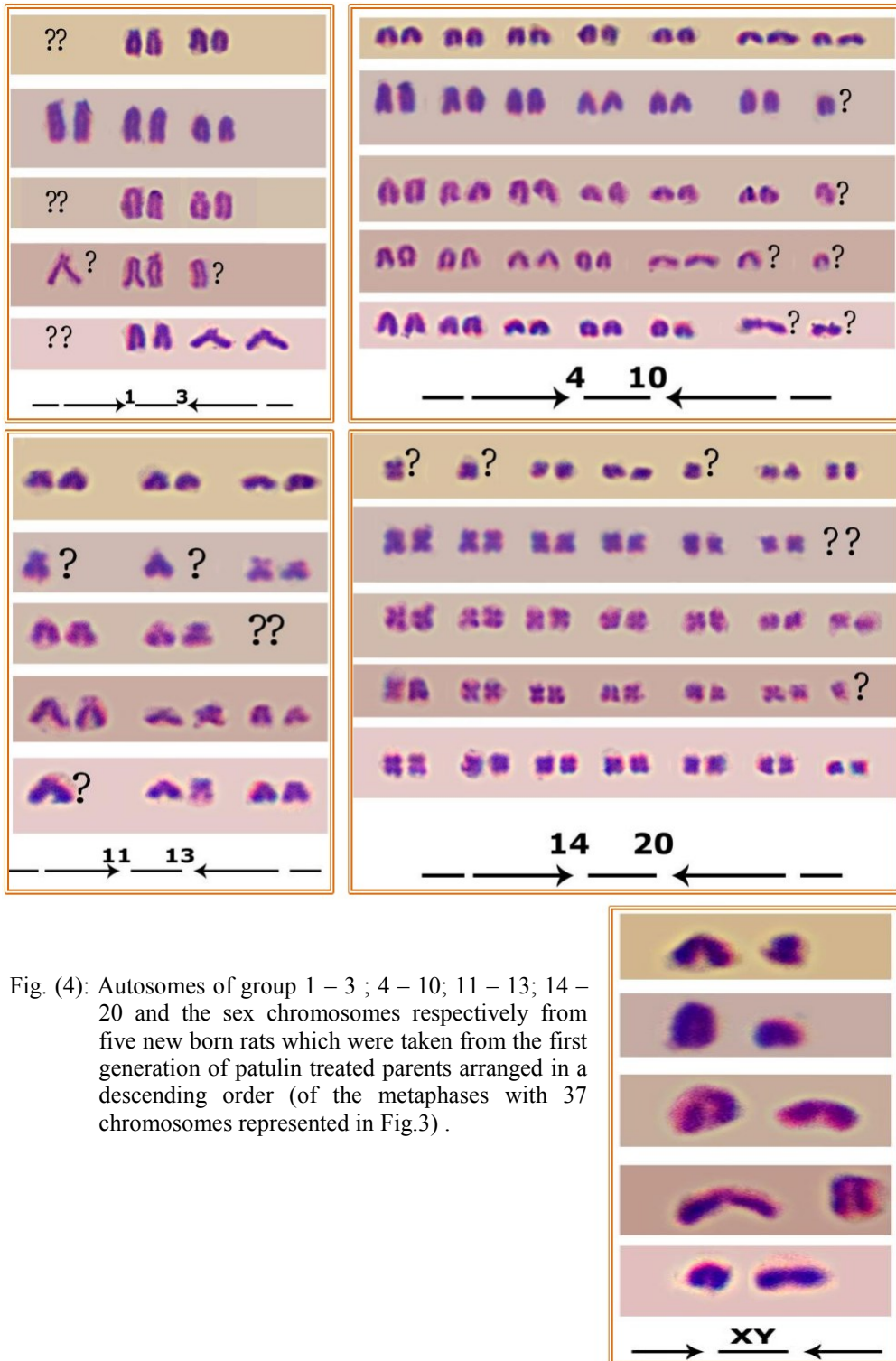


Fig. (4): Autosomes of group 1 – 3 ; 4 – 10; 11 – 13; 14 – 20 and the sex chromosomes respectively from five new born rats which were taken from the first generation of patulin treated parents arranged in a descending order (of the metaphases with 37 chromosomes represented in Fig.3) .

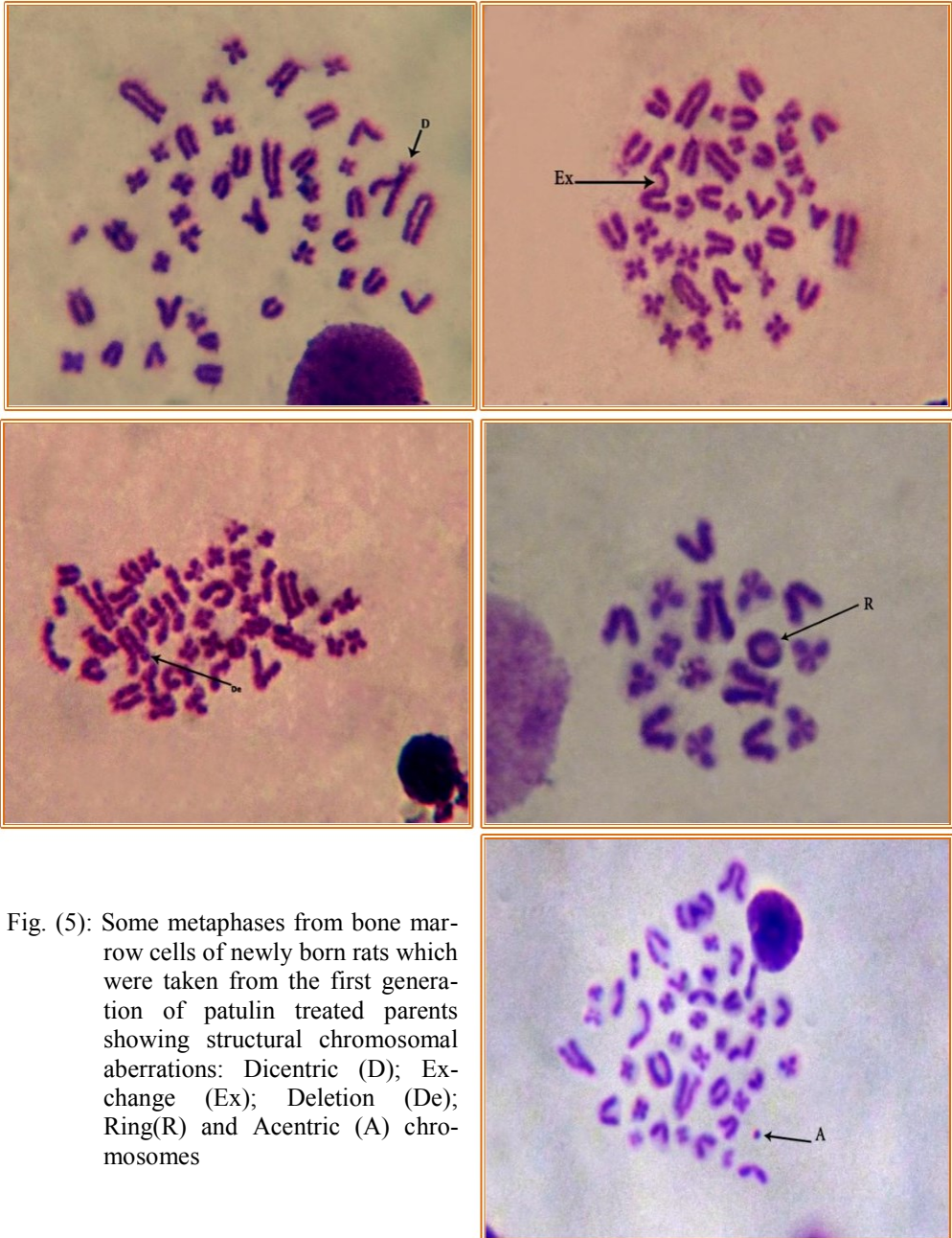


Fig. (5): Some metaphases from bone marrow cells of newly born rats which were taken from the first generation of patulin treated parents showing structural chromosomal aberrations: Dicentric (D); Exchange (Ex); Deletion (De); Ring(R) and Acentric (A) chromosomes

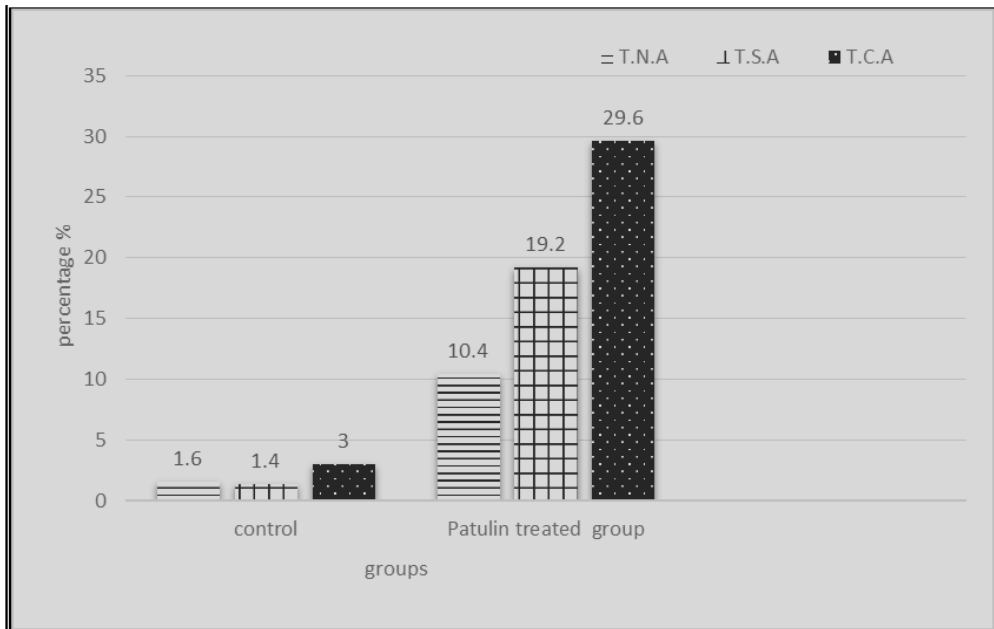


fig. ()ist gams ing t e percentage f t ta nume ica a e ati ns ()t ta stuctu a a e ati n ()an t ta c m s ma a e ati ns ()f m ne ma ce s f at s a ies ic e e ta en f m t e fi st gene ati n f patu in (0.002 mg/kg b.w.) treated parents.