

ANTIMUTAGENIC EFFECTS OF TANNIC ACID ON SOMATIC AND GERM CELLS OF RATS

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There is increasing interest in identification of naturally occurring substances that may be capable in diminishing genotoxicity of the environmental carcinogens and still serve as leads for the development of novel pharmacological agents. Many such medicinal plants have hepatoprotective, neuroprotective, anti-inflammatory and also antioxidant or radical-scavenging properties (Najmi *et al.*, 2010; Zhang *et al.*, 2010). Phenolic acids are secondary metabolites widely distributed in the plant kingdom and they come in the second priority after flavonoids in terms of their dominance. Tannic acid is a plant polyphenol which is found, along with other condensed tannins, in several beverages including red wine, beer, coffee, black tea, green tea, and many foodstuffs such as grapes, pears, bananas, sorghum, black-eyed peas, lentils and chocolate (Erexson, 2003; Bennett *et al.*, 2010). Similar to many polyphenols, tannic acid has been shown to possess antioxidant (Ferguson, 2001; Wu *et al.*, 2004; Andrade *et al.*, 2005), antimutagenic (Ferguson, 2001; Chen and Chung, 2000) and anticarcinogenic properties (Huang *et al.*, 2010; van der Logt *et al.*, 2003). The antioxidant mechanism of tannic acid is still far from being fully

understood; therefore, it requires further investigation. For example, in the presence of copper ions, tannic acid acts either as a prooxidant, promoting DNA damage (Ferguson, 2001; Khan *et al.*, 2000), or as an antioxidant, suppressing hydroxyl radical formation (Andrade *et al.*, 2005).

Tannic acid is always used as a food additive. Its safe dosage ranges from 10 to 400 mg, depending on the type of food to which it is added (Chen and Chung, 2000). Also, several authors have demonstrated that tannic acid and other polyphenols have antimutagenic and anticarcinogenic activities (Ferguson, 2001; Andrade *et al.*, 2005; Huang *et al.*, 2010; van der Logt *et al.*, 2003). Moreover, the consumption of polyphenol-rich fruits, vegetables, and beverages, such as tea and red wine, has been linked with inhibitory and preventive effects in various human cancers and cardiovascular diseases, which may be related-at least in part-with the antioxidant activity of polyphenols (Andrade *et al.*, 2005).

Phenolic acids have been shown to be anti-oxidant and it is a potent antagonist of the mutagenicity of polycyclic aromatic hydrocarbons (PAHs) such as

benzo(a)pyrene (BaP) (Mansour *et al.*, 2007). Benzo(a)pyrene is a semi-volatile, lipophilic, high molecular weight compound that belongs to the polycyclic aromatic hydrocarbon (PAH) family. Benzo(a)pyrene and other PAHs are products of combustion and can accumulate in crops via absorption from contaminated soils (ATSDR, 1995). Exclusive sources for BaP contamination of the environment and consequently human exposures include industrial and automobile emissions, hazardous waste sites, cigarette smoke, biomass burning, municipal incinerators, volcanic eruptions, home heating, and consumption of charcoal broiled and smoked foods (ATSDR, 1995; WHO, 1998). Food ingestion and inhalation are the major routes of entry into the human body for a large section of the general population exposed to PAHs (Ramesh *et al.*, 2004). The previous experimental data has shown that PAHs must be metabolically activated by peroxy radical dependent pathway and the electrophilic bay-region diol-epoxides act as the ultimate carcinogenic metabolites of PAH (Li *et al.*, 2004).

This study aimed to investigate the multifunctional antioxidant properties of tannic acid on BaP-induced genotoxicity in somatic and germ cells of rats.

MATERIALS AND METHODS

Chemicals

Benzo-a-Pyrene was purchased from Sigma Chemical Co. Thiobarbituric acid were obtained from Fluka (Berlin,

Germany). All other chemicals were of the highest analytical grades which were commercially available.

Experimental Animals

Fifty adult male Sprague-Dawley rats (100-120 grams) were used in this study. These animals were obtained from the animal house of the National Research Center, Giza, Egypt. The animals were housed in plastic cages, five per cage, and maintained on standard laboratory diet and water *ad libitum*.

Experimental design

Animals were divided into two experiments. The first experiment had twenty-five animals and was divided into five equal groups. Animals of the first group were orally treated by gavages with distilled water daily and used as control. Animals of the second group were treated with oral dose of TA (250 mg/kg/day) for 15 consecutive days according to Sehrawat *et al.* (2006). Animals of group three were injected intraperitoneally with BaP (175 mg/kg/day according to Hassan and Ahmed (2004) for two consecutive days. The fourth group received DMSO (1 mg/ml, i.p) for two consecutive days. Fifth group was given TA as before and followed there after by BaP challenge in the same dose regimen for two consecutive days. Twenty-four hours after the last BaP dose, animals were euthanized by cervical dislocation and samples of bone marrow were collected for the micronucleus assay. One of the testes were excised immediately and used for chromosomal analysis

in germ cells. The other one and the liver tissues were collected and stored at -80°C until use for determination of lipid peroxides level and nucleic acids contents.

The second experiment had twenty-five animals, divided into five equal groups, as follows: all animal groups were treated as those in the first experiment. However, all animals were sacrificed at the 35th day after the end of treatment for sperm analysis (Monesi, 1962).

I- Cytogenetic Analysis

I-a Micronucleus Test

Micronucleus test were analyzed according to Schmid (1975). The polychromatic erythrocytes (PCE) were screened for micronuclei and two thousand normochromatic erythrocytes (NCE) were screened to obtain the ratio of mitotic index. The reduction of the mitotic index was assessed on the basis of the ratio of PCE/NCE.

I-b Meiotic Chromosomes

The meiotic chromosomal preparations were made by the air drying technique (Brewen and Preston, 1987). Spermatoocytes at the diakinesis-metaphase I stages were examined for chromosomal aberrations including aneuploids, autosomal univalents, sex-univalents, polyploids and translocations.

I-c Sperm analysis

After 35 days of the last dose (duration of spermatogenesis), the animals of

the second experiment were sacrificed by neck vertebra luxation. The epididymides from each mouse were removed and sperm were collected as quickly as possible when each mouse was dissected and spermatozoa were counted using hemocytometer. Motility of the spermatozoa was evaluated under a light microscope. One drop of sperm suspension was placed on a glass slide, covered with a coverslip, and 10 random fields of view were examined. The number of motile and nonmotile sperm was counted. Motility was then expressed as the percentage of motile sperm to the total number of sperm.

For morphological abnormalities, a drop of a homogenate smeared on a cleaned slide was allowed to air drying and stained with approximately 0.05% aqueous eosin Y. The slides were coded and used for the examination of sperm head and tail abnormalities. For each animal, 500 sperms were examined according to the criteria of Jeong *et al.* (2005).

II - Evaluation of Nucleic Acids and MDA in Testis and liver

The frozen samples (liver and testis) were used for estimation of ribose nucleic acid (RNA), deoxyribose nucleic acid (DNA) and MDA.

II-a Estimation of Nucleic Acids

The method described by Bregman (1983) was used to determine the levels of nucleic acids. The sample tissues were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid

(TCA). After centrifugation, the pellet was extracted with ethanol. The levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

II-b Determination of lipid peroxides level

The method described by Ohkawa *et al.* (1979) was followed. The sample tissues were homogenized in TCA and the homogenate suspended in thiobarbituric acid. After centrifugation the optical density of the clear pink supernatant was read at 532 nm and the concentrations are expressed as nmol MDA/g tissue using extinction coefficient of 156.

III- Statistical analysis

The data was analyzed by analysis of variance (ANOVA). The differences among testing mean values in different groups was done by Duncan's multiple range tests (Waller and Duncan, 1969). SPSS 11.5 Window version was used for the statistical analysis. (Middle Books, 1977).

RESULTS AND DISCUSSION

BaP is a contaminant that occurs ubiquitously in the environment together with other PAHs as a product of incomplete combustion or pyrolysis of organic materials containing carbon and hydrogen.

Main sources of BaP and also PAHs in the environment are residential heating, industrial plants and cigarette smoke (Akpan *et al.*, 2006). Hepatotoxicity, nephrotoxicity, and hematologic alterations were among the major pathologies of BaP (Jung *et al.*, 2010; Tarantini *et al.*, 2009). Reproductive toxicity of the polyaromatic hydrocarbon has been investigated as well. However, data on testicular toxicity are scanty. In this sense, we have addressed the possible protective effects of the TA in such BaP-induced somatic and germ toxicity paradigm. In the present study the results of the micronucleus test (Table 1 and Fig. 1) revealed that animals treated with BaP exhibited a significant cytotoxicity in bone marrow as indicated by a marked reduction in the average number of PCEs (36.8 ± 1.46) as well as the ratio of PCEs/NCEs (0.59 ± 0.04). It is well established that BaP induces carcinogenic and mutagenic effects in mammalian and other animal cell systems (Jung *et al.*, 2010). BaP increased the different types of lesions in the DNA, including single and double strand breaks (DSBs), base damage as well as DNA cross-links (DNA-DNA and DNA-Protein), Delgado *et al.* (2008). Among these entire DNA, DSBs have been considered the critical lesion for the BaP-induced chromosome break (Cabaravdic *et al.*, 2010) and cell death (Jiang *et al.*, 2010). There was a correlation established between the induction of cell death and chromosomal aberrations and the frequency of micronuclei induction (Ayed-Boussema *et al.*, 2007). These micronuclei are formed from acentric fragment(s) or sometimes from the whole

chromosome (with defective kinetocore) (Zaichkina *et al.*, 2007). The cytogenetic damage induced by clastogenic agents on the mitotic cell is expressed as an increase in the micronuclei frequency during the interphase after the first post-treatment mitosis. Moreover, it is understood that non-repair/misrepair of the DNA double-strand breaks contributes to the chromosomal aberrations (Conrad *et al.*, 2011; See *et al.*, 2010) which could be analysed by the quantitative analysis of micronuclei. Therefore, the micronucleus assay is a very useful parameter used for assessing cytogenetic damage and is extensively used to screen the cytoprotective potential of synthetic and natural products (Mahrous *et al.*, 2002; Hassan and Ahmed, 2004).

Our studies demonstrated that TA was effective in counteracting the clastogenic effect of BaP as assessed by the inhibition of the induction of micronuclei by 83.3% (Fig. 3) which is correlated with the inhibition of chromosomal aberrations in spermatocytes (Table 2 and Fig. 2) in the same study as it markedly inhibited the total aberrations in spermatocytes by 88% (Fig. 3). In this concern, Hassan and Ahmed (2004) demonstrated that phenolic compound inhibited the induction of micronuclei and chromosomal aberrations in both somatic and germ cells. Numerous studies have suggested that phenolic compounds in plants constitute a major class of secondary plant metabolites with antimutagenic and antioxidant activities (Kaur and Kapoor, 2002). It has been also

shown that dietary supplementation of TA results in protection against the onset of forestomach, lung and skin neoplasia (Fresco *et al.*, 2010). Also, dermal application of TA showed a significant protection against cutaneous carcinogenesis (Delazar *et al.*, 2003).

The findings of the current study revealed that animals treated with BaP provoked marked deteriorations in epididymal functions. It reduced sperm count, motility and increased aberrations in the spermatocytes and sperm morphology (Tables 3 and 4). Actually, the data available on the effects of BaP on sperm parameters are scanty. Most of the data available are confined to the effects of BaP on testicular steroidogenesis and Sertoli cells. Inyang *et al.* (2003), Mahrous *et al.* (2002) and Hassan and Ahmed (2004) have demonstrated marked disruption in epididymal function as shown by progressive reduction in sperm motility following exposure to BaP in Fisher-344 (F-344) rats. It has also been recently reported that inhaled BaP significantly reduced testis weight and caused marked reductions in the components of steroidogenic and spermatogenic compartments of the testis in rats (Archibong *et al.*, 2008; Ramesh *et al.*, 2008). Similarly, Izawa *et al.* (2007) reported that diesel exhaust abundant in PAHs, to which BaP belongs, lowered notably the daily sperm production in four mice strains. By the same token, testis and epididymus weight, sperm motility, and daily sperm production rate were altered following exposure to PAHs (Jeng and Yu, 2008). Though the exact mechanism

underlying BaP-induced epididymal dysfunction in the current study is not known, it could be ascribed to its prooxidant effects in testicular tissue. The notion that BaP has shown earlier prooxidant potential (Regoli *et al.*, 2003) and the finding by Inyang *et al.* (2003) that free radicals generation could decrease sperm motility and lend support to this issue.

The data on sperms and testes chromosomes are in corroboration with the depletion of testicular nucleic acids were observed in the present study. The exact mode of action is not known, however; it might be related with biochemical changes observed in the testes, which showed an increase of MDA and depletion of nucleic acid content as shown in (Fig. 4) and (Table 5). However, Pretreatment with the TA before BaP improved the epididymal function as shown by the increased sperm count, motility, and decreased the level of spermatocyte and sperm morphology aberrations. As well as improving the nucleic acids content and decreasing the level of MDA in hepatic and testicular tissues (Fig. 4). This finding is coping with earlier reports that demonstrated protective effects of other flavonoids in testicular tissue following exposure to various toxicants (Goel *et al.*, 2006; Mersch *et al.*, 2006; Izawa *et al.*, 2007). Phenolic compounds work with enzymes and other antioxidants in the intestine, liver and lungs to prevent the activation of metabolic carcinogens (like BaP) before they damage DNA (VenKat, 2002). They could inhibit the cytochrome P450-

mediated metabolism of BaP into its ultimate mutagenic metabolite in such a way as to reduce its mutagenic potential (Chen and Yen, 1997).

In conclusion, the mechanism of protection in these studies seems to be inhibition of BaP metabolizing enzymes and their subsequent binding to DNA. The observed decrease in micronuclei, chromosomal aberrations and incidence of sperm abnormalities in animals receiving a pretreatment of TA before application of BaP suggest that inhibition in mutagenic effects of BaP overloaded animals may be due to preventing the activation of BaP before its binding with the DNA.

SUMMARY

Tannic Acid (TA) is a naturally occurring plant phenol shown to be present in normal human diet and have antimutagenic, anticarcinogenic and antioxidant activities. It is known to inhibit mutagenesis and carcinogenesis by polycyclic aromatic hydrocarbon. The aims of the current study were to evaluate the antimutagenic effects of TA against carcinogenic potential of Benzo-a-Pyrene (BaP) in somatic and germ cells of rats and investigate the role of biochemical changes, as a possible mechanism. The protocol included the oral treatment of rats with TA at dose of 250 mg/kg/day for 15 consecutive days and then rats treated with BaP at dose of 175 mg/kg for two consecutive days. The following cytogenetic experiments were conducted: (i) micronucleus test, (ii) meiotic chromosomes, (iii) analy-

sis of spermatozoa abnormalities also the quantification of nucleic acids and malondialdehyde (MDA) in hepatic and testicular cells were evaluated. Treatment with BaP increased the frequency of micronuclei in polychromatic erythrocytes (PCE) in the femora. It caused aberrations in chromosomes of testes and induced spermatozoa abnormalities. These changes might be attributed to the epigenetic mechanisms as revealed by an increase in concentrations of MDA and depletion of nucleic acids levels in both hepatic and testicular cells observed in the present study. Pretreatment of TA rendered protection against BaP induced DNA damage, as evidenced by inhibition of the frequency of micronuclei induction, chromosomal aberrations and sperm abnormalities by 83.3, 88.0 and 85.2% respectively. These inhibition percentage might be attributed to the antioxidant effects of TA as revealed by decreased the concentrations of MDA and improving nucleic acids levels in both hepatic and testicular cells observed in the present study. Overall, our results established an efficient antioxidant, anticlastogenic potential of TA, which may be of great pharmacological importance.

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Table (1): Effect of tannic acid and Benzo-a-Pyrene on the induction of micronuclei and the ratio of polychromatic to normochromatic erythrocytes from femoral bone marrow of rats.

Treatments	Mn-PCEs (Mean \pm SE)	PCEs % (Mean \pm SE)	NCEs % (Mean \pm SE)	PCEs/NCEs (Mean \pm SE)
Control	1.8 \pm 0.49c	52.8 \pm 2.56a	47.2 \pm 2.55c	1.14 \pm 0.12a
DMSO	3.2 \pm 0.37bc	45.8 \pm 2.08b	54.2 \pm 2.10b	0.86 \pm 0.07b
Tannic Acid (TA)	1.6 \pm 0.51c	52.4 \pm 1.25a	47.6 \pm 1.25c	1.11 \pm 0.05a
Benzo-a-Pyrene (BaP)	21.0 \pm 1.52a	36.8 \pm 1.46c	63.2 \pm 1.46a	0.59 \pm 0.04c
TA + BaP	5.0 \pm 0.32b	47.2 \pm 1.16ab	52.8 \pm 1.15bc	0.90 \pm 0.04ab

Five rats were used in each group. Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at $P < 0.05$.

Table (2): Effect of tannic acid and Benzo-a-Pyrene on chromosomal aberrations of spermatocytes in testis of rats.

Treatments	Structural Aberrations			Numerical Aberration		Total Aberration
	Autosomal-univalents	X-Y univalents	Chain	Aneuploids	Polyploids	
Control	1.2 \pm 0.37b	0.4 \pm 0.20c	0.2 \pm 0.20b	0.8 \pm 0.37b	0.6 \pm 0.40b	3.2 \pm 0.80bc
DMSO	1.2 \pm 0.35b	0.2 \pm 0.20c	0.2 \pm 0.20b	0.6 \pm 0.25b	0.8 \pm 0.37b	3.0 \pm 0.32bc
Tannic Acid (TA)	0.6 \pm 0.25bc	0.2 \pm 0.20c	0.0	1.2 \pm 0.37ab	0.4 \pm 0.25b	2.4 \pm 0.68c
Benzo-a-Pyrene (BaP)	4.6 \pm 0.51a	4.8 \pm 0.37a	2.0 \pm 0.32a	2.2 \pm 0.35a	3.0 \pm 0.32a	16.6 \pm 0.40a
TA + BaP	1.8 \pm 0.37b	1.6 \pm 0.40b	0.2 \pm 0.20b	0.6 \pm 0.40b	0.6 \pm 0.24b	4.8 \pm 1.0b

Five rats were used in each group. Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at $P < 0.05$.

Table (3): Effects of different treatments on epididymal sperm count, motility and total abnormality percentage.

Tested Parameters	Control	DMSO	Tannic Acid (TA)	Benzo-a-pyrene (BaP)	TA+BaP
Sperm count $\times 10^6$	37.0 \pm 1.60a	36.4 \pm 1.30a	39.6 \pm 0.51a	21.8 \pm 1.10c	33.4 \pm 1.63b
Motility (%)	90.0 \pm 1.58a	85.0 \pm 1.60b	93.0 \pm 1.22a	70.0 \pm 1.56c	86.0 \pm 1.87b
Abnormal sperms (%)	1.04 \pm 0.17c	1.36 \pm 0.25c	1.12 \pm 0.15c	9.96 \pm 0.27a	2.36 \pm 0.29b

Means with different superscripts (a, b, c, d) between groups in the same row are significantly different at $P < 0.05$.

Table (4): Effects of Tannic Acid and Benzo-a-Pyrene on epididymal sperm abnormalities.

Treatments	Tail Ab-normality	Head Abnormalities				Total Ab-normalities
		Amorphous	Triangle	Banana	Without-hock	
Control	2.6 ± 0.51c	1.2 ± 0.37c	0.4 ± 0.25b	0.60 ± 0.24b	0.4 ± 0.24b	5.2 ± 0.86c
DMSO	2.6 ± 0.60c	1.4 ± 0.51c	1.0 ± 0.45b	1.0 ± 0.45b	0.8 ± 0.37b	6.8 ± 1.24c
Tannic Acid (TA)	1.8 ± 0.37c	1.6 ± 0.25c	0.40 ± 0.24b	0.60 ± 0.25b	1.2 ± 0.37b	5.6 ± 0.75c
Benzo-a-Pyrene (BaP)	14.4 ± 1.36a	14.6 ± 0.93a	6.4 ± 0.51a	8.2 ± 0.37a	6.2 ± 0.34a	49.8 ± 1.35a
TA + BaP	5.2 ± 0.66b	4.4 ± 0.92b	0.8 ± 0.20b	0.40 ± 0.24b	1.0 ± 0.32b	11.8 ± 1.46b

Means with different superscripts (a, b, c, d) between groups in the same column are significantly different at $P < 0.05$.

Table (5): Effects of Tannic Acid and Benzo-a-Pyrene on Nucleic Acid contents in hepatic and testicular tissue of rats.

Treatments	Hepatic Tissue (Mean ± SE)		Testicular Tissue (Mean ± SE)	
	DNA (mg/gm)	RNA (mg/gm)	DNA (mg/gm)	RNA (mg/gm)
Control	0.316 ± 0.010a	0.189 ± 0.003a	0.283 ± 0.015a	0.190 ± 0.02a
DMSO	0.295 ± 0.008a	0.157 ± 0.008b	0.263 ± 0.008a	0.167 ± 0.01a
Tannic Acid (TA)	0.325 ± 0.013a	0.169 ± 0.012ab	0.286 ± 0.010a	0.192 ± 0.03a
Benzo-a-Pyrene (BaP)	0.211 ± 0.005b	0.092 ± 0.006c	0.175 ± 0.003b	0.186 ± 0.04a
TA + BaP	0.321 ± 0.009a	0.181 ± 0.006a	0.288 ± 0.009a	0.191 ± 0.01a

Means with different superscripts (a, b, c) between groups in the same column are significantly different at $P < 0.05$.

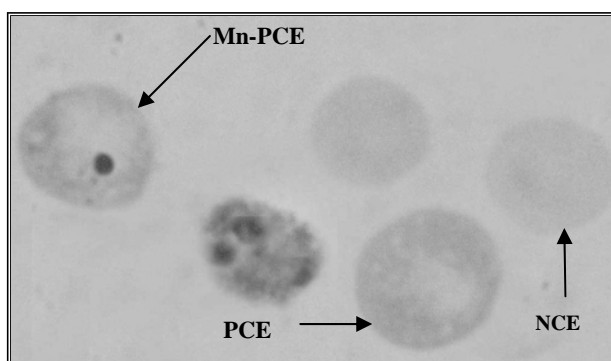


Fig. (1): Showing polychromatic erythrocyte (PCE), micronucleated polychromatic erythrocyte (Mn-PCE), and normochromatic erythrocyte (NCE).

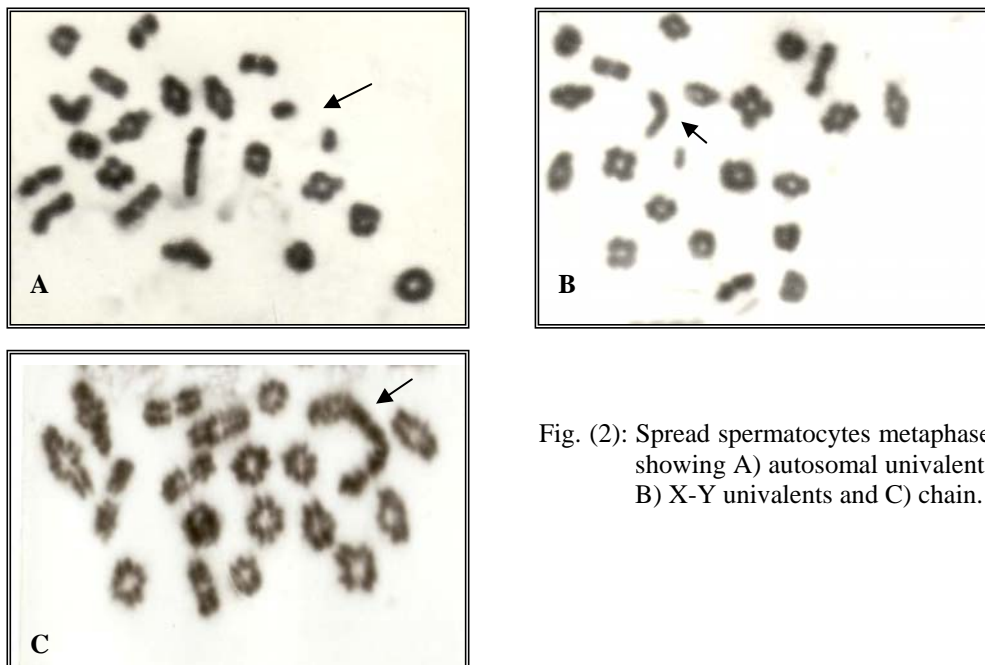


Fig. (2): Spread spermatocytes metaphases showing A) autosomal univalents, B) X-Y univalents and C) chain.

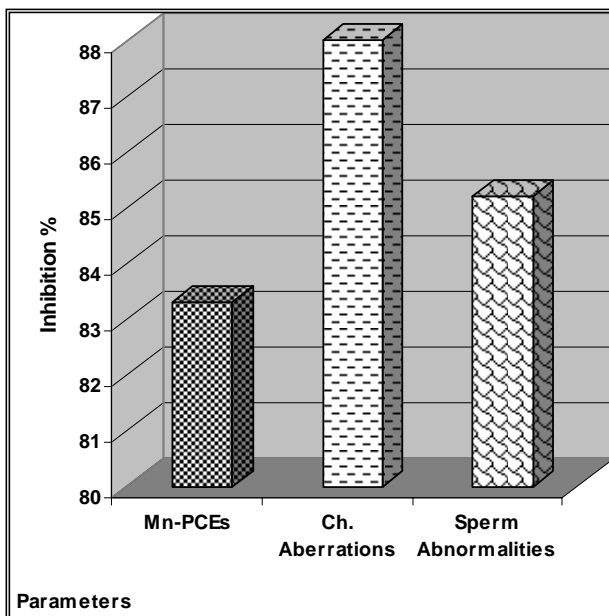


Fig. (3): Histogram showing inhibition effects of Tannic Acid on micronuclei incidence, chromosomal aberrations and sperm abnormalities induced by Benzo-a-Pyrene.

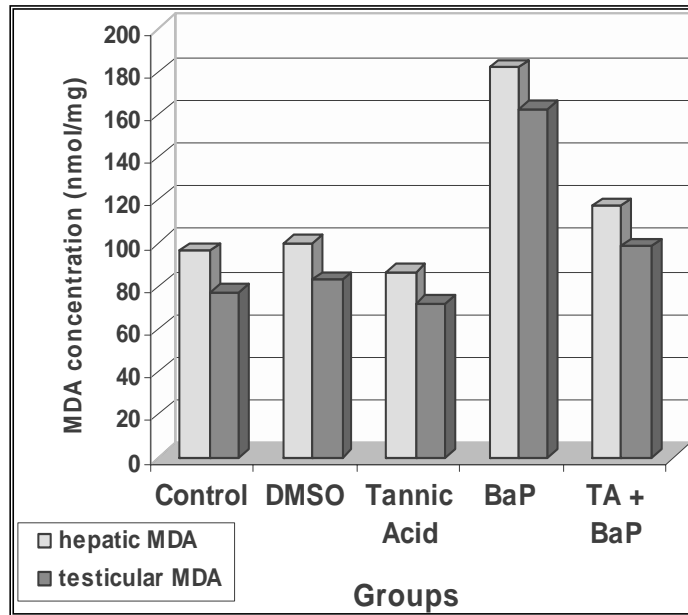


Fig. (4): Histogram showing the effects of Tannic Acid (TA) and Benzo-a-Pyrene (BaP) on MDA content in hepatic and testicular tissues in rats