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Cytogenetic and Pathological Studies on The Effect of Gibberellic Acid in Rabbit

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Abstract

This study was designed to clarify the effect of the plant growth hormone, gibberellic acid (GA3) on the cytogenetic and pathological profile of adult male rabbits. Thirty bucks were classified into 3 equal groups; a control and two other treated groups; the first (indirectly exposed group, IEG) was forced to feed on a previously sprayed green fodder (alfalfa) with the recommended dose of GA3, while the second (directly exposed group, DEG) was forced to drink ad libitum on 75 ppm GA3 in water for 30 successive days.

Both exposed groups evoked a significant increase in the total aberrated cells and total chromosomal aberrations of bone marrow cells, however, the most predominated chromosomal aberrations were deletions, ring chromosomes, end to end associations and aneuploidy, in addition to DNA damage using comet test. The second treated group (DEG) showed a significant increase in the percentage of these aberrations not only comparing to the control but also with the first treated group (IEG). Pathological changes in the liver, kidneys, lungs and testes were reported in both treated groups. These changes were severe in the second treated group (DEG). After one month recovery period, the deviated parameters of the first treated group (IEG) were nearly returned to the normal values, meanwhile, the second treated group (DEG) still revealed significant changes compared to either the control or the first treated group (IEG).

From this study, It could be concluded that GA3 has a genotoxic and cytotoxic effects. These effects were severe in rabbits directly exposed to the hormone (DEG) compared to those fed on sprayed green fodder with its recommended dose (IEG).

Introduction

Gibberellic acid (GA3) is a plant growth regulators (**Çelik, et al., 2002)** and widely used in Egypt, to increase the growth of fruits and vegetables **(Kamel, et al., 2009)**. If gibberellic acid or one of its metabolites is applied to dwarf varieties of peas, broad beans or maize, growth is greatly accelerated **(Jones, 1973).** Although GA3 is used commonly in agriculture, its potential hazardous effects on human health were relatively unexplored **(Sakr, et al., 2003).** Little is known about the cytogenetic and pathological effect of GA3 on animals. Gibberellic acid caused pathological, immunohistochemical and biochemical changes in the kidney of young

mice that increased with duration of exposure planned with oxidative stress with some sort of self recovery after stoppage of exposure **(Mona and Wafaa 2010).**

The cytogenetic effect of different concentrations of gibberellin A3 (0.1, 0.5, 1 and 2 mg) was studied in human lymphocyte culture. Treating cultures with gibberellin induced chromosomal aberrations, sister chromatid exchanges and DNA damage. The chromosomal aberrations included gap, break, deletion and centromeric attenuation. DNA damage was also detected by comet assay (**Sakr, et al., 2009**). Similarly, gibberellic acid was found to induce chromosomal aberrations in human lymphocytes **(Zalinian, et al., 1990)** and mice **(Bakr, et al., 1999).**

GA3 has a genotoxic and cytotoxic effects. The increase in the genotoxic effect corresponds to a decrease in the mitotic activity of bone marrow cells (**Nassar, et al., 2012).** It induced a strong and obvious DNA damage, in addition to hepatonephrotoxicity represented in liver fibrosis, fatty metamorphosis and necrosis with kidney interstitial fibrosis which appears as segmental and diffuse glomerular sclerosis and tubulointerstitial injury **(Hanan, et al., 2010)**. Nephrotoxicity with a significant decrease in the antioxidant enzyme activities in kidneys of suckling pups and their mothers during late stage of pregnancy and early post natal periods were also recorded **(Troudi, et al., 2011).**

Moreover, testicular changes including Leydig's cell degeneration, reduction in seminiferous tubules and necrotic symptoms and sperm degeneration were recorded in GA3 treated rats **(Hanaa, et al., 2013).** However, two week withdrawal period did not ameliorate the negative pathological effects in different organs of two weeks old broiler chicks fed on GA3 containing diet (25-125 ppm) for two weeks **(Abdelhamid, et al., 1994).**

This study aimed to evaluate the cytogenetic and pathological changes that may be associated with direct or indirect exposure to the plant growth hormone, gibberellic acid in adult male rabbits.

Materials and Methods:

Materials:

1-Animals:

Thirty sexually mature male California rabbits weighing 2.5-3 kg were used. Rabbits were kept in the laboratory under constant temperature ($24\pm2^{o}C$) for at least one week before and throughout the experimental work. They were maintained on a standard diet and water ad libitum.

2-Gibberellic acid (GA3):

Berelex tablet weighing 10 gm contains 10% GA3 and manufactured by Valent BioSciences Corporation. One tablet Berelex^{R}10% dissolved in 80 liter water was enough to spray 8 kerate alfalfa as recommended by the manufacturing company.

Methods:

Animals were divided into 3 equal groups; a control group and two other treated groups. The first (indirectly exposed group, IEG) was forced to feed on a previously sprayed alfalfa one week after spraying with the recommended dose of GA3 similar to that applied in the agricultural field. Meanwhile, the control and the second treated group (directly exposed group, DEG) were fed on non sprayed alfalfa allover the day hours with a standard concentrated diet for all groups at the night hours. However, the second (directly exposed group, DEG) was forced to drink *ad libitum* 75 ppm GA3 **(Hanan, et al 2010)** in water for 30 successive days. Five rabbits from each group were slaughtered after thirty days of treatment period, while the other five were slaughtered after another thirty days recovery period.

Chromosomal aberration assay:

Chromosomal analysis of the bone marrow cell was carried out according to the technique modified by **Carbonell, et al. (1996).**

Comet assay (single strand breaks of DNA): After 30 days of treatment and after 30 days recovery period, rabbits were slaughtered immediately and slices of liver tissues were immediately taken, washed with phosphate buffer saline then kept at-80 for determination of DNA damage by alkaline comet assay according to **Singh, et al. (1988).** The migration of damaged DNA was evaluated by observing and measuring the nuclear DNA, where the rounded spot of DNA was considered as a normal DNA spot, while the nuclear DNA migrating towards the anode appeared as comet spot and considered as damaged DNA spot. Spots of DNA were examined and classified into three types: (1) normal spots; round shape, (2) damaged spots; in which the length of the migrated fragments is less than or equal to the diameter of the basal nuclear DNA, and (3) strongly damaged spots; where the length of the comet was greater than the diameter of the basal nuclear DNA.

Pathological examination: Careful postmortem examination was carried out on scarified rabbits of all groups, then tissue specimens of liver and kidneys, lung and testes from control and treated animals were fixed in 10% neutral formalin, dehydrated, embedded in paraffin wax and sectioned at 5 μ to be stained with H&E for light microscopic examination **(Bancroft and Gamble, 2002).**

Statistical analysis was carried out according to **Snedecor and Cochran (1976).**

Results and Discussion

Gibberellic acid (GA3) is one of the plant growth regulators used in Egypt on a wide range of vegetables and fruits. Animals may be exposed to residues of GA3 in diet or drinking water **(Tolmin, 2004).** The present study was designed to evaluate its genotoxic effect manifested by the induction of chromosomal aberrations or DNA damage and its cytotoxic effect manifested by the pathological alterations in the tissues of liver, kidneys, lung and testes.

The normal chromosome number of rabbits was found to be 2n= 44,xy. Different types of chromosomal aberrations were observed in both treated groups with gibberellic acid, however, a significant increase in total aberrated cells and total chromosomal aberrations were recorded in this study (Table, 1). These results are in accordance with **Sakr et al. (2009)** who reported that Gibberellic acid was found to induce chromosomal aberrations in human lymphocyte cultures. Similarly, **Bakr et al. (1999) and Nassar et al. (2012)** reported a significant increase in the incidence of total chromosomal aberrations in bone marrow cells of albino mice and rats, respectively, subjected to gibberellic acid. The author explained these chromosomal aberrations on the basis of the effect of gibberellic acid on DNA synthetic system during the S-phase of the earlier cell cycles making it more drastic. The most predominated chromosomal aberrations were deletion (d), ring chromosomes (R), end to end association (EE), and aneuploidy (Fig. 1,2,3,4). These data refer to the interaction of GA_3 with DNA leading to deletions in the terminal end of chromosomes or chromatids, this deletion causes instability of chromosomes or chromatids, then lead to end to end associations and ring chromosomes. These events may lead to mitochondrial membrane depolarization and ended by apoptosis **(Abou-Eisha 2001),** However, apoptosis was also recorded in the liver and testes by flow cytometric analysis due to the administration of GA3 to rats **(Hanaa et al, 2014).** The second treated group (DEG) showed a significant increase in the percentage of these aberrations not only comparing to the control but also with the first treated group (IEG). After one month recovery period, the total chromosomal aberrations and the total aberrated cells showed significant increase in the DEG only but not in the IEG compared to the control (Table, 1)

In addition, the mitotic index, which gives information about the frequency of mitotic cells (mitotic activity) showed a significant increased in DEG at the end of treatment period as well as after one month recovery. Similar data were recorded by **Jovtchev et al. (2010)** who attributed the increase in the frequency of chromosomal aberrations and micronuclei in bone marrow cells of rats to the increase in the mitotic activity of these cells. The mitotic index is a useful and simple method for analysis of cell proliferation **(Rudolph et al., 1998).** Despite showing an elevation during normal growth of animals, as well as cellular repair at the site of an injury, the significant increase in mitotic index indicates more cells are dividing, and thus obvious in cancer cells **(Urry et al., 2014)**, however, it may aid in the prognosis of some tumers as patients with high mitotic index had shorter disease-specific survival **(Ha et al, 2016)**.

The single cell gell electrophoresis (SCGE), known as comet assay, is recognized as one of the most sensitive and reliable test available for DNA strand break detection with the advantage of being fast, simple and applicable for any eukaryotic cell type in vivo as well as in vitro **(Kumaravel and Jha, 2006 and Speit**

and Hartmann, 2006). The percent of DNA in the tail reflecting the amount of DNA migrated out of nucleus is strongly recommended as the parameter of choice and directly linked to DNA break frequency **(Dogan, et al., 2011).** DNA detection by comet assay showed that strong damage spots of DNA (migration towards the anode) were observed in both treated groups, but reported to be more pronounced in DEG (Fig. 5). Evaluation of DNA damage via comet assay was recorded in this study (Table, 2) and revealed a significant damage of DNA as indicated by the significant increase in tail lengh (px) and tail DNA % in both treated groups with gibberellic acid which were more pronounced in the second treated group, however, one month recovery period was enough to repair the DNA damage in IEG but not in DEG. These results came in accordance with **Abou-Eisha (2001)** who reported that gibberellic acid induced a significant increase in the level of DNA breakage in human blood cells in a dose dependant manner. Similarly, **Hassab-Elnabi and Sallam (2002)** and **Saker et al (2009)** recorded an increase in the number of damaged cells and the damaged DNA spots in human lymphocyte culture by increasing the concentration of GA3. The DNA damage may be attributed to the direct attack of DNA by gibberellic acid causing alkali labile and single strand breaks (alkylating the DNA bases) followed by total genomic damage, or may be due to accumulation of nucleases as reported by **Fath et al. (1999).** The mechanism of gibberellic acid to induce DNA damage may be also attributed to elevation of oxidative stress markers **(Abou-Eisha 2001).** These strand breaks may be repaired, resulting in no persistent effect, may be lethal to the cell, or may be fixed into a mutation resulting in a perminant viable changes. They may also lead to chromosomal damage which is also associated with many humen diseases including cancer **(OECD/OCDE, 2016).** On the other hand, **Macgregor (1988)** demonstrated that GA3 was essentially nontoxic by various routs of applications for different animal species. These differences may be attributed to the dose and its duration, the route of administration, and /or the sensitivity of the species to GA3.

Regarding the pathological study, gross examination revealed different degrees of congestion in all examined organs with some atrophy in the testes of both treated groups which was more pronounced in recovered DEG. However, the present work revealed that GA3 induced different histopathological changes in the liver, kidney, lung and testes. All the tested organs of the treated rabbits showed an inflammatory response in the form of congested blood vessels, interstitial hemorrhage with degenerative changes and leucocytic infiltration. Liver of post treated IEG showed mild congestion with periductal fibrosis and edema **(Fig. 6)**, while liver of post treated DEG showed perivascular lecuocytic cells infiltration with congested blood vessels **(Fig.7)** and liver of recovered DEG showed necrosis of some hepatic cells with multiple pyknotic nuclei represented in pyknosis **(Fig. 8).** These changes became prominent in the DEG. The same changes were found in the

liver of albino rats treated with GA3 **(Sakr, et al., 2003).** They concluded that inflammatory reactions were considered as a prominent response of the body tissue facing any injurious impacts. However, chronic inflammation plays an important role in creation of oxidative stress **(Erin et al., 2008)** which produces the imbalance between the production of free radicals and antioxidant defence system resulting in tissue damage **(Oruc and Uner, 2002)**.

Our findings agree with those obtained by **Sakr, et al. (2003)** who found masses of inflammatory leucocytic infiltrations in several areas of liver with congestion of central vein. Hyperplasia of bile ducts and finally severe cytoplasmic vacuolization associated with degenerative changes varied from karyolysis to severe karyorrhexis and complete pyknosis of nuclei were also recorded in rats treated with 24 ppm GA3 by gavage in 0.2 ml saline, 3 times a week for 3 weeks. These changes attributed to the fact that liver is the first target organ in toxicological prospects regarding to detoxification, biotransformation and excretion of xenobiotic so after enteric uptake of injurious materials, it is the first organ to be exposed to such hazards via the portal circulation.

Kidney of treated DEG showed focal area of cystic dilation of some renal tubules **(Fig. 9)** and that of treated IEG showed hypocellularity of some glomeruli **(Fig. 10)** in agree with **Mona and Wafaa (2010)** who reported extensive tubular dilatation with wide lumen, thin epithelium, desquamated cells and hyaline casts in the lumen of some tubules in male albino rats treated with the same dose of GA3 in water. Similar changes were found in the kidney of mice due to other plant regulators **(Yazar and Baydan, 2008)** which may be attributed to the imbalance between the production of free radicals and antioxidant defence system resulting in tissue damage **(Oruc and Uner, 2002)**. However, Lung of treated IEG showed congestion of pulmonary blood vessels **(Fig. 11)**, while lung of treated DEG showed peribronchial mononuclear cells infiltrations **(Fig. 12)**, in addition to red hepatisation of pulmonary tissue **(Fig. 13)** as an advanced stage of pneumonia due to action of GA3 on lung with the increase duration of treatment.

Testis of treated IEG showed congestion of testicular blood vessels **(Fig. 14A)**, while testis of recovered IEG showed limited germinal stages developed activities **(Fig. 14B)**, in addition to normal testicular tissue with very minimal degenerative changes **(Fig. 15)**. Testes of GA3 treated rabbits also showed disturbances in spermatogenesis process including degenerative changes in the testes with severe disintegration of spermatocytes, spermatogenic arrest with moderate tubular necrosis, and Leydig's cell degeneration. In addition, there was reduction in the size of the seminiferous tubules with some spermatogonia detached from the basement membrane, a result that came in accordance with **Hanaa, et al. (2013)**. These pathological changes in the testes may be attributed to the direct testosterone-like action of GA3, which inhibits testosterone secretion **(Hanaa, et al., 2013)**,

however, **Moussa and Abdel-Hafez (1983)** found that ALP activity was decreased which proposed to have a role in the synthesis of testosterone, and is necessary for the early stages in spermatogenesis. The reported pathological changes proved to be time dependent in IEG while showed severe progress in case of DEG manifested in atrophy of seminefrous tubules with destruction of interstitial tissue **(Fig. 16)** in addition to the reported seminoma in the testes **(Fig. 17A&B)**. Seminoma was recorded in one of the 5 rabbits of DEG, one month after the end of treatment period. This result, agree with **Erin et al. (2008)** who reported that chronic GA3 consumption in animals increased tumor formation and **El-Mofty, et al. (1994)** who showed that gibberellin A3 induced breast and lung adenocarcinomas in mice when administered for 22 months. The recorded seminona in this study, may be correlated to the elevated mitotic index which indicates more cells are dividing, and thus obvious in cancer cells **(Urry, et al., 2014)** or attributed to the reported DNA damage through replication of DNA with carcinogen-induced lesions which is an essential step in the initiation of carcinogenesis **(Williams and Weisburger, 1991).**

This study concluded that GA3 has a genotoxic and cytotoxic effects. These effects were severe in rabbits directly exposed to this plant hormone (DEG) compared to those fed on sprayed green fodder with its recommended dose (IEG). However, the deviated parameters were nearly returned to their normal values after one month recovery period in the IEG but not in the DEG.

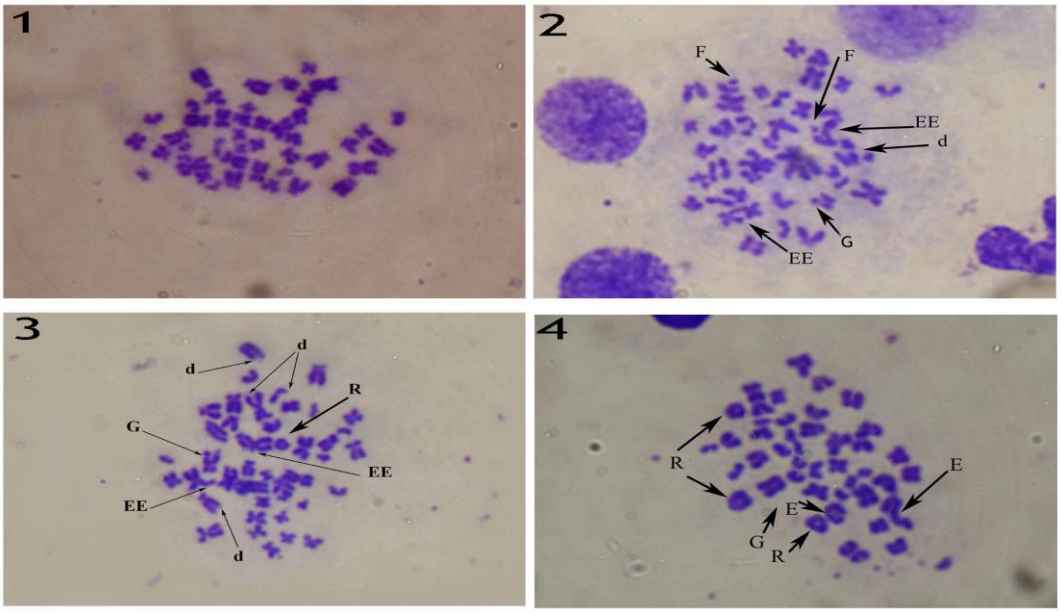

Fig (1-4): A metaphase spread of adult male rabbit intoxicated with gibberellic acid showing
Fig 1: Normal chromosome number (2n=44,xy) and profile.
Fig 2: chromatid deletion (d), chromatid gap (G), end to end association (EE) and fragment (F).
Fig 3: Chromatid deletion (d), end to end association (E), Chromosome gap (G) and ring (R).
Fig 4:Ring chromosome (R), end to end association (E) and chomatid gap (G).

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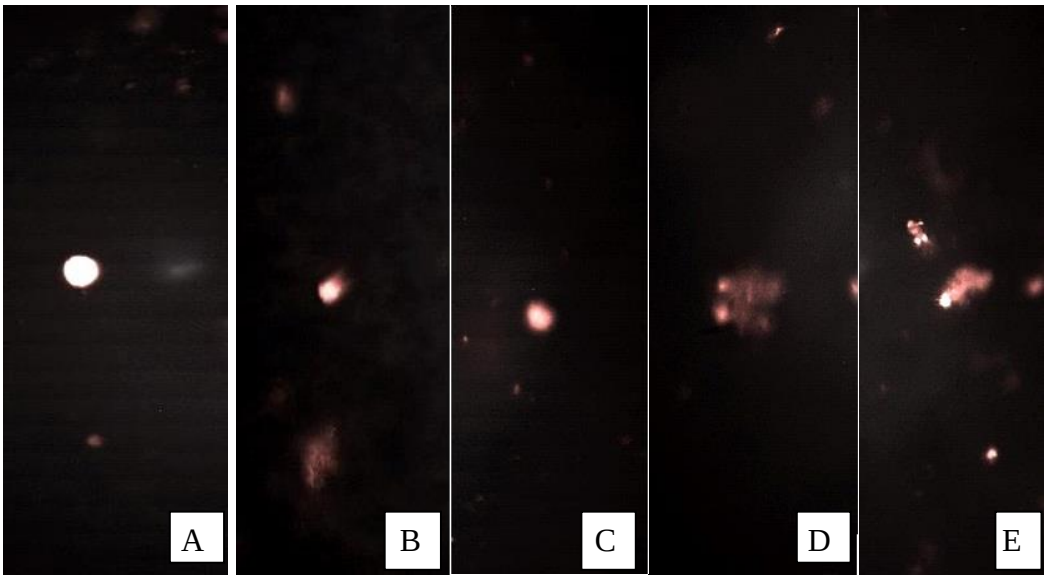

Fig. 5: Nucleus of liver cells of control and treated rabbits with gibberellic acid showing:
(A) Normal DNA spot with the absence of tail (no migration) in control group.
B) Damaged DNA spot with small tail (migration towards the anode) in IEG at the end of treatment.
C) Damaged DNA spot with small tail (migration towards the anode) in IEG after one month recovery. D) Strong damaged DNA spots with long tail (more migration towards the anode) in DEG at the end of treatment.
E) Strong damaged DNA spots with long tail (more migration towards the anode) in DEG after one month recovery.

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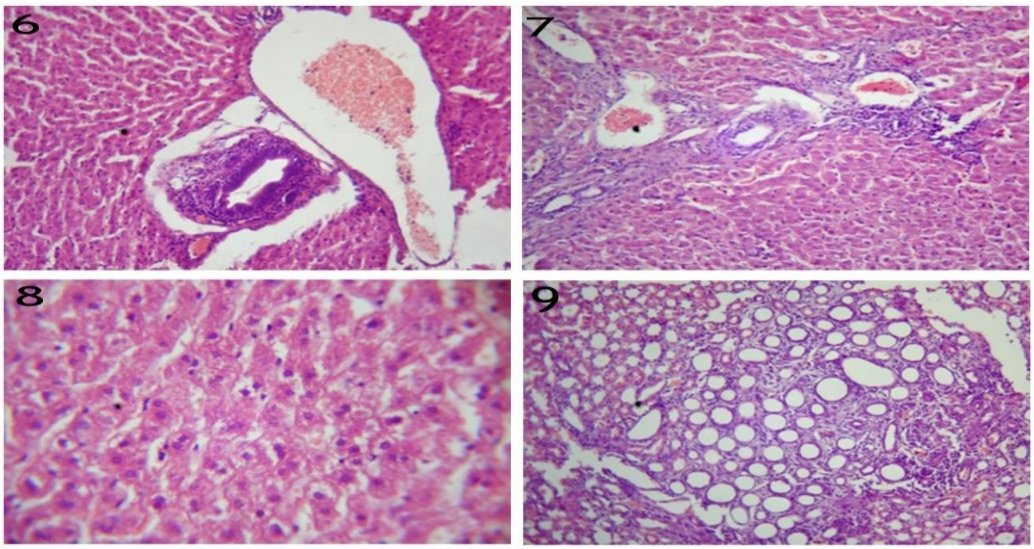

Fig. (6-9): pathological sections of GA3 treated rabbit showing:

Fig. 6- Liver of post treated IEG showing mild congestion with periductal fibrosis and edema. (H& E x 200).

Fig. 7: Liver of post treated DEG showing perivascular leucocytic cells infiltration with congested blood vessels . (H& E x 100).

Fig. 8- Liver of recovered treated DEG showing necrosis of some hepatic cells with multiple pyknotic nuclei. (H& E x 400).

Fig. 9- Kidney of post treated DEG showing focal area of severe cystic dilatation of some renal tubules . (H& E x 100).

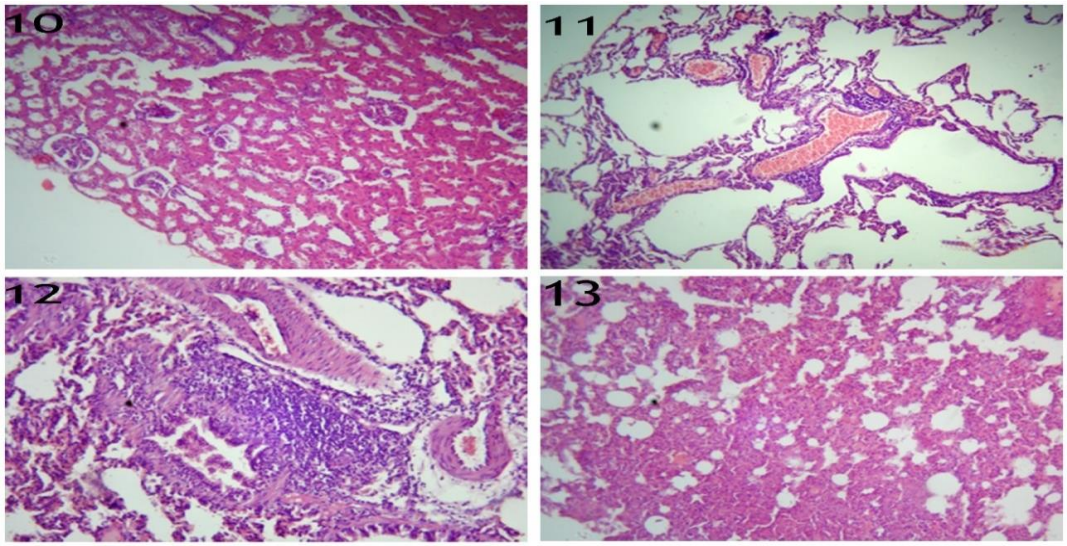

Fig.(10-13): pathological sections of GA3 treated rabbit showing:

Fig. 10-Kidney of treated IEG showing hypocellularity of some glomeruli. (H& E x 100)

Fig. 11-Lung of post treated IEG showing congestion of pulmonary blood vessels . (H& E x 100).

Fig. 12- Lung of post treated DEG showing peribronchial mononuclear cells infiltrations . (H& E x 200).

Fig. 13- Lung of treated DEG showing red hepatisation of pulmonary tissue. (H& E x 100)

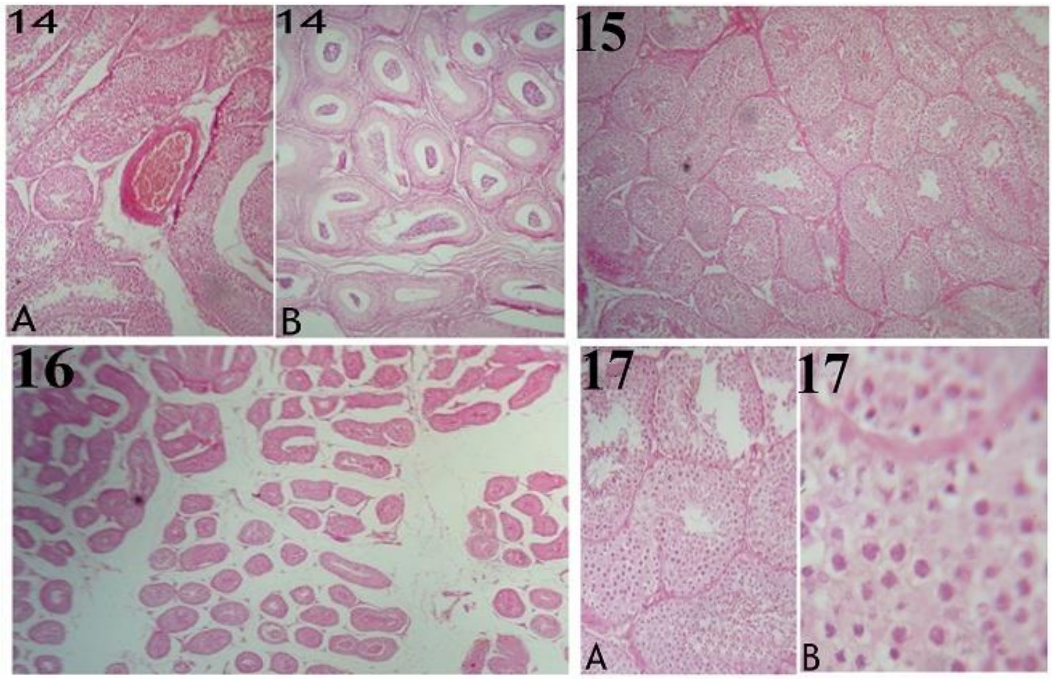

Fig.(14-17): Testis of GA3 treated rabbit showing:

Fig. 14(A)- Testis of post treated IEG showing congestion of testicular blood vessels. (H& E x 200).

Fig. 14(B)-Testis of recovered IEG showing limited germinal stages developed activities. (H& E x 100).

Fig. 15- Testis of recovered IEG showing normal testicular tissue with very minimal degenerative changes. (H& E x 200).

Fig. 16- Testis of post treated DEG showing atrophy of seminefrous tubules with destruction of interstitial tissue. (H& E x 100).

Fig. 17(A)- Testis of recovered DEG showing early stage of seminoma focal replaced testicular parenchyma . (H& E x 200).

Fig. 17(B)- Testis of recovered DEG showing high power of previous figure to show seminoma. (H& E x 400).

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Table(1): Effect of direct (DEG) or indirect (IEG) exposure of adult male California rabbits to gibberellic acid on chromosomes

Time of assay	Group	Mitotic index %	Total aberrated cells	Structural chromosomal aberrations%					Numerical %	Total Chromosomal aberrations%
				CA	Gap	Detetion	Ring	EE	Peridiploidy	
One month treatment (first assay)	control	3.61 ± 0.65^{b}	1.6 ± 0.75^{b}	0.4 ± 0.4^{b}	-	0.4 ± 0.4^{c}	0.4 ± 0.4^{c}	-	0.8 ± 0.49^{b}	2.0 ± 0.89^{d}
	IEG	4.04 ± 0.59^{b}	29.0 ± 1.89^{a}	0.4 ± 0.4^{b}	0.8 ± 0.49	15.2 ± 1.09^{b}	5.6 ± 0.81^{b}	6.2 ± 0.8^{a}	4.4 ± 0.98^{a}	32.6 ± 1.54^{b}
	DEG	3.92± 0.45^{b}	31.4 ± 3.28^{a}	2.0 ± 0.63^{a}	1.2 ± 0.8	27.4 ± 8.46^{a}	9.2 ± 0.97^{a}	5.8 ± 0.58^{a}	5.2 ± 0.73^{a}	41.8 ± 2.5^{a}
One month recovery (second assay)	control	3.74± 0.68^{b}	2.4± 0.4^{b}	1.2 ± 0.49^{ab}	-	0.4 ± 0.4^{c}	0.4 ± 0.4^{c}	-	0.4 ± 0.4^{b}	2.4 ± 0.4^{d}
	IEG	7.22± 1.02^{a}	6.4± 0.75^{b}	0.4 ± 0.4^{b}	0.4 ± 0.4	3.6 ± 0.5^{c}	2.0 ± .63^{c}	2.0 ± 0.89^{b}	1.0 ± 0.45^{b}	9.4 ± 1.02^{c}
	DEG	6.73± 1.23^{a}	29.0 ± 2.19^{a}	0.8 ± 0.49^{ab}	1.2 ± 0.49	20.2 ± 1.96^{ab}	6.6 ± 0.68^{b}	6.6 ± 0.4^{a}	3.8 ± 0.8^{a}	39.2 ± 1.71^{a}

Values with different litters within the same column differed significantly at $p< 0.05$

Table 2: Oxidative DNA damage (comet assay) observed in liver of rabbits after direct or indirect exposure to GA3 (Mean±S.E.):

Time of assay	Group	Tailed cells %	Head diameter(px)	Tail length(px)	% DNA in tail
Control		13 ± 0.55^{c}	59.8 ± 0.8^{a}	12.4 ± 0.24^{c}	12 ± 0.55^{d}
One month treatment (first assay)	IEG	20.4 ± 0.93^{b}	58.8 ± 0.73^{a}	15.2 ± 0.37^{b}	14.2 ± 0.37^{c}
	DEG	21.2 ± 0.73^{b}	57.6 ± 0.68^{a}	18.8 ± 0.58^{a}	16.8 ± 0.37^{b}
One month recovery (second assay)	IEG	19.2 ± 0.58^{b}	48.6 ± 0.81^{b}	13.4 ± 0.51^{c}	14.6 ± 0.87^{c}
	DEG	24.6 ± 0.51^{a}	36.4 ± 0.6^{c}	19.4 ± 0.51^{a}	21.6 ± 0.75^{a}

Values with different litters within the same column differed significantly at $p< 0.05$

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