

Genetic and Ultrastructural studies in bone marrow and testis of mice cells under the effect of Glurenor drug

Sekena, H. Abd El-Aziem and Aziza, M. Hassan
Department of Cell Biology, National Research Centre

Abstract

Mutagenetic effects and ultrastructural changes of antidiabetic Glurenor drug were studied in vivo in the highly sensitive micronucleus test.

Eight groups of forty male mice used in this study to detect chromosomal abnormalities in somatic and germ cells. Three groups orally administrate therapeutic doses of Glurenor at (30, 60, 120) mg/kg daily the fourth group act as control group. Control group and the treated group with 120 mg/kg were used for ultrastructural examination. While the other four groups were used for bone marrow micronucleus test which receives successive doses of Glurenor as (0.0 & 30) mg/kg for 1, 10 & 20 days. A cytogenetic examination of treated and untreated mice showed a significant increase of total chromosomal aberrations ($P < 0.01$) in both somatic and germ cells at dose 120 mg/kg. when compared by control group. Also Glurenor induced micronucleus polychromatic erythrocytes. Glurenor revealed ultrastructural changes in liver cells represented by deep condensation in the nucleus which revealed by the appearance of a large gap around the nucleus. The condensed chromatin appeared as large sharply marginated electron dense mass that a butted on the nuclear envelope. In addition to electron dark distanced elsewhere (prenuclear), swollen endoplasmic reticulum, Also, observed enlarged nucleolus, and thickening the nuclear membrane. Large vacuoles "hydropic degeneration" also was noticed in the cytoplasm.

Our studies had the objective of examining experimentally whether the supposed mutagenic effect of Glurenor can be demonstrated and verified by methods of mutagenicity testing using experimental mammals.

In conclusion, this study revealed that Glurenor gave a positive reaction with a clear dose response in mice. Glurenor gave a mutagenic response from of the chromosomal aberration in somatic and germ cells as well as in micronucleus test. Glurenor showed ultrastructural changes in the liver cells of mice.

Key words: Glurenor, chromosomal aberrations, polychromatic erythrocytes (PCEs), ultrastructural, liver

Introduction

Glurenor is one of sulphonylureas (to lbutamide, chlorpropamide, libenclam-ide, glipizide, gliclazide, glimepiride) that stimulate pancreatic insulin release.

Sulfonylureas stimulate insulin secretion from pancreatic cells and are widely used in the treatment of type 2 diabetes (*Ashcroft, and Ashcroft 1992*).

They commonly used in the reduction of blood sugar levels. Sulfonylureas work

by stimulating insulin production, sensitizing insulin receptors, and inhibiting the production of glucose by the liver.

The sulfonylurea drugs lower plasma glucose concentrations in diabetic patients by stimulating insulin secretion and by potentiating the biologic effect of the insulin on tissues such as skeletal muscle, fat and liver. The mechanism of the latter so-called extra-pancreatic effect may be

activated by increasing the deficient numbers of insulin receptors on muscle, fat or liver cells (*Skillman and Feldman, 1981*). Also, sulfonylurea drugs showed that glypentine induces a significant decrease in total lipid and cholesterol values (*Lopez et al., 1976*). Side effect with sulfonylurea therapy are rare and include dermatological hypersensitivity, gastrointestinal discomfort, and vasomotor symptoms (most frequently reported with chlorpropamide) (*Lebovitz, 1990*). On the other hand *Ferner (1988)* suggest that chlorpropamide is the most toxic sulfonylurea but glyburide causes dangerous hypoglycemia as often as chlorpropamide. While the sulfonylureas, troglitazone can cause serious adverse events, their incidences are low and can be minimized by strict adherence to the prescribing guidelines and close monitoring of treated patients *Charles and Clark (1998)*. Also, sulphonylureas showed cytogenetic changes (*Abd EL-Azeim et al.,2002; Mahrous and Kamal 1995*).

This study presents the results of an in vivo cytogenetic investigation of Glurenor drug in order to evaluate any increase in the number of chromosome aberrations or micronucleus polychromatic erythrocytes or any change in liver cell produced compared with controls as an indication of mutagenic activity.

Material and Methods

drug used

Glurenor (gliquidone 30 mg tablets, manufactured by MINAPHARM Egypt under licence of A.MENARINI Italy) the active ingredient is gliquidone. Glurenor is an oral drug of sulfonylurea class.

Animal and Treatments

Forty male adult Swiss albino mice aged (10-12) week old and weight 20-25gm. were used and divided into 8 groups each of five mice. Animals were obtained from a closed random bred colony at animal house of National Research Centre, Doki, Giza, Egypt. The animal room was maintained on a 12 h light/dark cycle, the temperature range was 20 to 24°C, and the humidity range was 60 to 70%. Mice were

fed standard breeding granulated diet, and water was supplied ad libitum.

The animals were orally given the tested drug at specified doses calculated according to *Paget and Barnes 1964*. and classified into:-

Micronucleus assays groups

1. control group
2. single dose of Glurenor (30 mg/kg).
3. 10 successive doses of Glurenor.
4. 20 successive doses of Glurenor.

Chromosomal analysis groups

1. control group
2. therapeutic dose (30 mg/kg).
3. two fold of therapeutic (60 mg/kg).
4. four fold of therapeutic (120 mg/kg).

Experimental design for chromosomal abnormalities and micronucleus test:

After 24 hours from the last administration the animal injected intraperitoneally (i.p.) with 0.5 ml of 0.05% of colchicines 2 hours. before sacrifice. The femora and testis were removed from animals and the micronucleus, chromosomes were prepared as follows.

For micronucleus analysis, slides were prepared according to *Salamone et al. (1980)*. The bone marrow cells were mixed with one drop of fetal calf serum and smeared on clean glass slides. Slides were allowed to air dry before methanol fixation at room temperature for 10 min. Slides were stored at .20 .C° in a sealed box. The sides stained with 5 % Giemsa and mounted with DPX. Two thousands PCEs/ animal were recorded.

Chromosomal analysis

After one week of treatment the animals were injected intraperitoneal (i.p.) with colchicines 0.5 ml of 0.05 % mg/kg. two hr. before sacrifice to arrest the cells of bone marrow in metaphase. The chromosome were prepared and slides were stained with Giemsa. Chromosomal aberrations were scored following the guide line of *Preston et al. (1987)*. The aberration frequencies per cell for chromatid, chromosome and mitotic index were calculated. The testis of the same animals were removed and slides were prepared according to *RUSSO (2000)*. One hundred metaphase spreads per animal were

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examined in bone marrow and seventy five metaphase spreads were examined in spermatocytes.

Statistical Analysis

Comparison of reliability analysis (F. test) was done statistical significance between the treated and control groups of mice according to *Snedecor and Cochran (1961)*.

Electron microscopy:

Liver specimens of animals which treated with 120 mg/kg. and control groups were removed from living animals to avoid the shock reaction. Specimens were immediately fixed by immersion in 4 % gluteraldehyde in 0.1 M sodium cacodylate buffer, PH 7.3 for 6 hours (*Gupta and Berridge, 1966*). Then post fixation was carried out in 1 % osmium tetroxide in the same cacodylate buffer for 2 hours (*Palde, 1952*). Specimens following dehydration were embedded in pure resin (1:3 araldite: epoxy). Ultrathin sections were mounted on copper grids and stained with uranyl acetate (*Stempex and Ward, 1964*) and lead citrate (*Reynolds, 1963*). Stained sections were investigated under JEOL 100 S Transmission Electron Microscope of Ain Shams University, at 80 KV accelerating voltage.

Results

In the screening test, adult male mice were given a dose of Glurenor that corresponded to 4 times the therapeutic dose. In

this way chromosomal aberrations in somatic and germ cells showed Glurenor to be positive in both tests (TableS 2&3). To verify these results, different doses of Glurenor was administered, and dose-response relationships determined.

Cytogenetic Results

Effect on polychromatic erythrocytes (PCE)

Table (1) shows the induction micronucleated polychromatic erythrocytes (Mn PCEs) in bone marrow cells of animals treated with Glurenor. The results indicate that there was non-significant difference between the control and treated animals with single dose (30 mg/kg)of Glurenor. Repeated treatment (oral administration) with drug for 10 &20 consecutive days showed significant increase in Mn PCEs as $P < 0.05$ & 0.01 respectively. Fig (1) shows polychromatic erythrocytes with micronuclei (arrow).

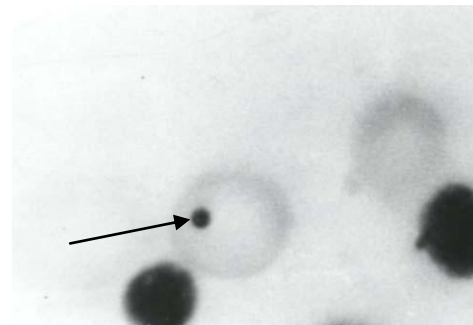


Fig.(1): Photograph showing micronuclei in polychromatic erythrocytes

Table (1): the mean value of micronuclei detected in polychromatic erythrocytes of bone marrow cells induced by Glurenor in male mice

Doses	Number of examined cells	Number of animal	M ±S.E
0.0 mg/kg	10000	5	2.4 ± 0.67
30 mg/kg	10000	5	3.2 ± 0.74
300 mg/kg	10000	5	5.8 ± 0.66 *
600 mg/kg	10000	5	6.8 ± 0.66**

* $P < 0.05$

** $P < 0.01$



Table (2): Mean value of different chromosomal aberrations induced by Glurenor in male mice

Doses	break M ± SE	delation M ± SE	fragment M ± SE	Ring M ± SE	E.E M ± SE	C.F M ± SE	C. A M ± SE	Endo M ± SE	Total M ± SE	gap M ± SE	Polyploidy M ± SE
0.0 mg/kg	0.0 ± 0.0	0.2 ± 0.20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.24	0.6 ± 0.40	1.2 ± 0.20	0.4 ± 0.24	0.4 ± 0.24
30 mg/kg	0.4 ± 0.24	0.8 ± 0.37	0.6 ± 0.24	0.2 ± 0.20	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.24	0.6 ± 0.24	3.2 ± 0.58*	0.8 ± 0.37	0.6 ± 0.24
60 mg/kg	0.6 ± 0.24	1.2 ± 0.37	0.8 ± 0.20*	0.4 ± 0.24	0.2 ± 0.20	0.2 ± 0.20	0.8 ± 0.20	0.6 ± 0.24	4.6 ± 0.67*	0.8 ± 0.37	0.8 ± 0.20
120 mg/kg	1.2 ± 0.37*	1.4 ± 0.24**	1.2 ± 0.20**	0.6 ± 0.40	0.4 ± 0.24	0.2 ± 0.20	1. ± 0.32	0.6 ± 0.40	6.4 ± 0.51**	1.2 ± 0.37	1.2 ± 0.58

E.E = end to end associations
 C.F = centric fusions
 C.V = centromeric attenuations
 Endo = endomitosis

Effect of Glurenor on bone marrow cells

The present study indicated that oral administration of different doses of Glurenor to male mice induced structural and numerical chromosomal abnormalities. Structural chromosomal aberrations were noticed as gaps; breaks; deletions; fragments; ring; end to end associations; centric fusion; endomitosis and centromeric attenuations. While, numerical changes were noticed as polyploidy.

Table (2) represented the mean values of different structural aberrations and polyploidy induced by Glurenor in bone marrow cells of male mice.

Results showed that Glurenor oral administration caused statistically significant increase in the frequency of total

structural chromosomal aberrations at all experimental doses (3.2 ± 0.58 , 4.6 ± 0.67 & 6.4 ± 0.51) when compared with control (1.2 ± 0.20). this increase was found to be statistically significant at ($P < 0.05$, 0.05 & 0.01) respectively even after exclusion of gaps.

In moderate doses, fragments were the most frequent type of abnormality observed, it was increased significantly as ($P < 0.05$) compared with control. In high doses treated groups a significant increase in breaks & deletions were noticed as $P < 0.05$ & 0.01 respectively compared with control groups Fig.2, 3 .

chromosomal abnormalities detected in spermatocytes in the form of chains (Fig.5), autosomal univalent, X-Y univalents (Fig 4), aneuploid and

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polyploidy (Fig 5) there were a significant increase as ($P < 0.05$, 0.05 & 0.01) respectively in total structural abnormalities in spermatocyte cells with different doses of the drug. There was a significant increase as ($P < 0.05$) in the frequency of the ploidy

in spermatocyte cells with low doses treated animals and highly significant increase of ploidy with other doses as ($P < 0.001$) as compared with control. while anuploidy showed increase but non-significant in all treated groups.

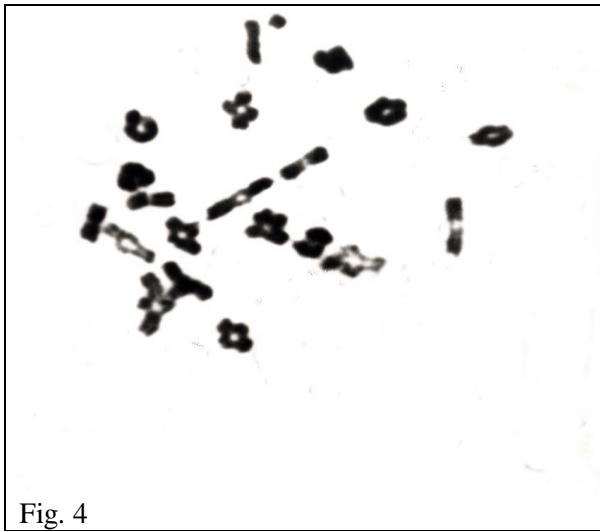


Fig. 4

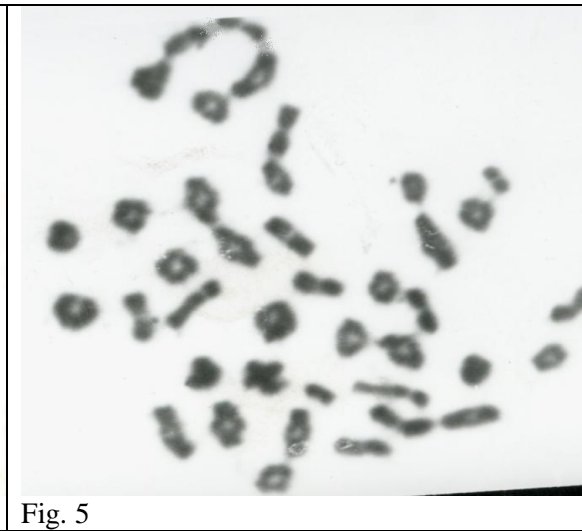


Fig. 5

Table (3): Mean values of different aberrations induced by Glurenor drug in spermatocyte of mice

Doses	X-Y	Auto	Chain	Total	Ploidy	N-1
	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE	M ± SE
0.0 mg/kg	0.2 ± 0.20	0.2 ± 0.20	0.0 ± 0.0	0.4 ± 0.24	0.2 ± 0.20	0.4 ± 0.24
30 mg/kg	1.4 ± 0.51*	1.0 ± 0.45	0.4 ± 0.24	2.8 ± 0.97*	1.8 ± 0.37*	1.2 ± 0.37
60 mg/kg	2.0 ± 0.32*	1.4 ± 0.51*	0.6 ± 0.24	4.0 ± 0.84*	2.8 ± 0.37**	1.4 ± 0.51
120 mg/kg	3.4 ± 0.51**	2.6 ± 0.51*	1.6 ± 0.51*	7.6 ± 0.40**	3.4 ± 0.51**	2.2 ± 0.37**

Auto = autosomal univalents

X-Y = x-y univalents

* $p < 0.05$

** $p < 0.01$

Ultrastructure examination:

Ultrastructurally examination of liver tissue showed irregularly dilated rough, swollen & degranulated endoplasmic reticulum in the hepatocytes of treated groups (Fig.7). Nuclei of most of the affected cells appeared swollen with irregularly shaped envelop and uneven

distribution of chromatin material and often with peripherally distributed heterochromatin. Also, hypertrophied nucleolus. Large vacuoles "hydropic degeneration" were common in the cytoplasm (Fig.8). The cytoplasm of hepatocytes appeared devoid of glycogen in comparison with control.

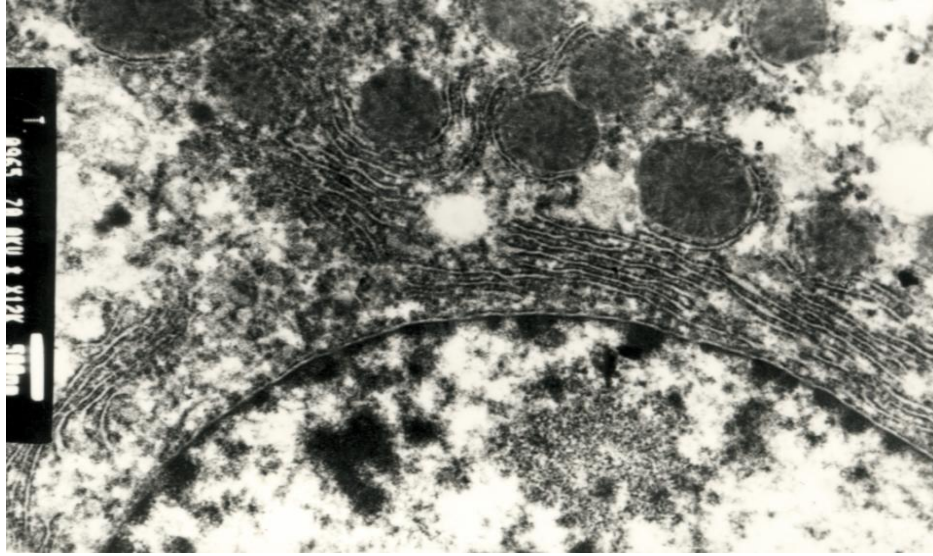


Fig. 6: E.M. photomicrograph of hepatocyte of control groups showing nucleus with its nuclear membrane and chromatin rough endoplasmic reticulum and mitochondria. X. 12000

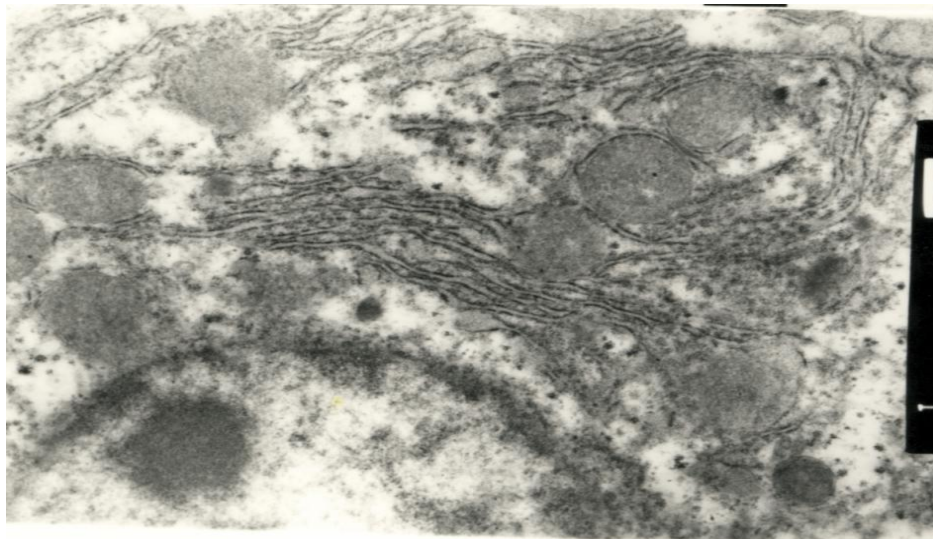


Fig. 7: E.M. photomicrograph of hepatocyte of treated group (120 mg/kg) that was revealed by appearance of large gap around the nucleus. The condensate chromatin showed large sharply margined electron dense mass that a butted on the nuclear envelope (arrow). Presence of areas of cytoplasmic lysis. Electron dark inclusions present else where (prenuclear). X. 12000

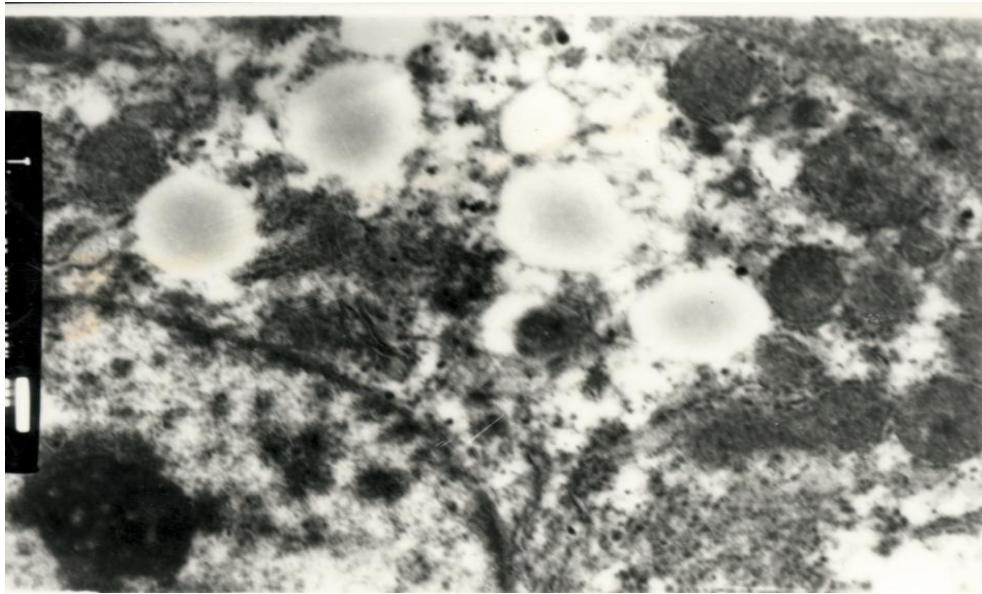


Fig. 8: E.M. photomicrograph of hepatocyte of treated group (120 mg/kg) hypertrophied nucleolus with thickening nucleolar membrane and large vacuoles (V) "hydropic degeneration". X. 10000

Discussion

Sulfonylurea drugs used on a large scale for the treatment of type 2 diabetes. The present study was conducted on one drug of this group called Glurenor to detect its mutagenic side effect. The results showed that the drug produced abnormalities in chromosomal aberrations in both somatic cells and germ cells of mice. In addition, the drug induces micronuclei in polychromatic erythrocytes cells in bone marrow. Similar observations were recorded by *Kulkarni et al. (1985)* who decided that there was a statistically significant increase in the individual numbers of sister chromatid exchanges per metaphase in each of the patients treated with chlorpropamide of the pooled control values. Also, *Abd EL-Azeim et al. (2002)* which found that oral treatment of either Amaryl or Diaben (sulfonylurea drugs) induce chromosomal aberrations in somatic and germ cells of mice. Similar observations were recorded by *Endo and Ingalls (1968)* who found that diabetes drugs (Alloxan and Streptozotocin) induced chromosomal anomalies such as gaps, breaks, polyploidy and aneuploidy in tissues from defective fetuses of diabetic mothers of mice. Similar observations were reported also by *Koller (1973)*. The

obtained results were found to be in agreement with the study of *Watson et al (1976)* cultured lymphocytes of diabetics treated with chlorpropamide drug. In addition, *Cole and Trasler (1980)* recorded that insulin induced a significant reduction in neuroectoderm mitotic index of mice and cell proliferation. Also, *Brown and Wu (1977)* showed that chlorpropamide (sulfonylurea drugs) induced sister chromatid exchanges in Chinese hamster cells. Chlorpropamide and tolbutamide induced chromosomal aberrations either in mice or Chinese hamster (*Renner and Munzner 1980*). Sulfonylurea agents have teratogenic effects in animal as reported by *Mahrous & Kamal (1995)* in this study on the metformin and tolbutamid (antidiabetic drugs) have genotoxic effect in the pregnant rats and their fetuses. Also, *El Nahas et al. (1988)* reported that both alloxan and streptozotocin diabetic drugs induced a high frequency of severe scoliosis in combination with intrauterine growth retardation and skeletal abnormalities in fetus and these diabetic drugs induced significant chromosomal aberrations in bone marrow of pregnant mice. The authors suggested that both drugs interfere with synthesis of DNA congenital abnormalities.

These suggestions support our findings about the side effects of Glurenor on chromosomes in somatic and germ cells.

On the other hand *Baster et al. (1982)* reported that chlorpropamide, did not induce structural chromosomal aberrations under *in vivo* conditions in human lymphocytes. While, *Djelic (2001)* noticed that insulin stimulates mitotic division and has not exhibited genotoxic properties under experimental conditions when studied sister chromatid exchange and micronuclei cultured human lymphocytes treated with insulin.

The present results suggested that sulfonylurea agent act directly on DNA as there appear to be no likely mechanisms where it could react with or damage a DNA molecule directly since it binds strongly to serum protein, it may produce its effects indirectly by affecting enzyme function.

Few previous work had been done on the ultrastructural pattern of liver following treated sulfonylurea drugs.

In this study ultrastructural examination of liver treated sulfonylurea drugs showed different alterations were significantly more intensified. Areas of cytoplasm were observed to be deprived of normal cellular organelles, The present study showed swollen degranulated endoplasmic reticulum, Free ribosomes were seen in abundance. Similar results observed by *Reaven et al. (1973)* when studied the effect of induced diabetes mellitus and insulin therapy replacement on hepatic ultrastructure and protein synthesis in rats. He found that increase in hepatocyte Golgi very low density lipoprotein (VLDL) content, but only a small increase in estimates of VLDL-TG secretion rate (post-Triton WR 1339 increment in plasma TG level). Although these findings are consistent with the thesis that VLDL-TG synthesis and secretion are increased 24 h after administration of Streptozotocin.

The present results revealed nuclei of most of the affected cells appeared swollen with irregularly thick nuclear envelop and uneven distribution of chromatin material and often with peripherally distributed heterochromatin Also, enlarged nucleolus. While *Nepomnyashchikh et al. (2001)*

showed that degenerative changes in liver biopsy specimens from diabetic patients with diabetes included alteration of hepatocyte nuclei, and formation of crescent nuclei..

Our results revealed many large vacuoles "hydropic degeneration" in the cytoplasm. Similar observation was noticed by *Dai et al. (2001)* when study the effect of carbon tetrachloride in liver of rats. A minimal amount of glycogen was observed in hepatocytes in comparison with control. Similar results revealed by *Neupert et al. (2003)* in hepatocytes of precision-cut rat liver slices after incubation for 24 and 48 hours. they observed an organelle-free layer of fine granular material in the apical cytoplasm followed by parallel oriented stacks of rough endoplasmic reticulum near by. The cytoplasm of parenchymal cells consisted of defined areas of clear cytoplasmic material containing numerous branching tubular profiles of smooth endoplasmic reticulum, presumably in the regions with depleted glycogen aggregates. Dilated endoplasmic reticulum free of ribosomes and clumping of chromatin in the nucleus of hepatocytes.

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دراسة التغير الوراثي و التركيب الخلوي الدقيق في نخاع عظام وخصية
الفئران تحت تأثير عقار جلورينور
سكينة حسانين عبد العظيم و عزيزة محمد حسن
(قسم بيولوجيا الخلية- المركز القومي للبحوث)

يتناول هذا البحث دراسة تأثير المعالجة بواحد من أحدث العقاقير المخفضة لسكر الدم المرتفع على الفأر الأبيض الصغير.

لقد أجرى البحث على أربعين ذكر من ذكور الفئران البيضاء البالغة تم تقسيمها إلى ثمان مجموعات، أربع منها جرعت بعقار جلورينور بالجرعة المسموح بها لمدة (1، 10، 20) يوما وأخذت المجموعة الرابعة كمجموعة ضابطة وذلك لإكتشاف و تحديد نسبة الخلايا المحتوية على فجوات في نخاع العظام.

وقد استخدمت الأربع مجموعات الأخرى لتحديد الاختلافات الكروموسومية في خلايا نخاع العظام والخصية حيث تم تجريع ثلاث منها بجلورينور كالنالي (30، 60، 120) مجم/كجم من وزن الجسم وذلك لمدة أسبوع وأخذت الرابعة كمجموعة ضابطة. و قد أخذت عينات من الكبد كمثل للخلايا الجسدية لدراسة التغيرات في التركيب الدقيق لهذه الخلايا عند استخدام الجرعة 120 مجم/كجم و مقارنتها بالمجموعة الضابطة.

لقد أظهرت الدراسة أن دواء جلورينور له تأثير وراثي ضارحيث يزيد من نسبة الأختلالات الكروموسومية في خلايا نخاع العظام والخصية وأيضا نسبة الفجوات وأن هذه الزيادة تظهر بوضوح في الجرعة العالية فقد ظهرت في خلايا نخاع العظام زيادة ذات دلالة احصائية نتيجة للمعاملة في كل من الكسور الكروماتينية و الإستطالة أما بالنسبة لخلايا الخصية فقد ظهرت فيها كل من أختلال السلسلة و انفصال الكروموسوم الجنسي و الجسدي و التضاعف في عدد الكروموسومات.

ولقد تغيرت الصورة الخلوية للتركيب الدقيق لخلايا الكبد فقد ظهر السيتوبلازم وبه ريبوزومات متناثرة و فجوات و ظهرت الشبكة الاندوبلازمية منتفخة و يوجد في السيتوبلازم أيضا أجسام غيمية وحببيات جليكوجين. أما في النواه فقد زاد حجم الكتلة الكروماتينية و كأنها أوشكت أن تتحلل.

و توصى هذه الدراسة بالحد من إستخدام عقار جلورينور لما له من تأثير وراثي و خلوي ضار.