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RESEARCH ARTICLE

MOLECULAR DIAGNOSTIC STUDIES ON THE MUTAGENICITY OF AN ANTIEPILEPTIC DRUG IN MICE FETUS

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Abstract

The aim of the present study is to determine the effect of antiepileptic drug (Phenytoin) on the mitochondrial CYT b gene in mice fetus. The used mice were arranged in four different groups: (G1) control group, (G2) treated males with untreated females, (G3) treated females with untreated males and (G4) treated males with treated females. All treated groups were interperitoneally injected with a dose of phenytoin 8mg/kg for 15 days before mating .The injection continuously with pregnant females at 21^{th} day of gestation,. At delivery the fetuses are collected .In this study, the molecular techniques investigate the effect of the drug (in each group) on CYT b gene of mice fetus at delivery by PCR, RFLPs and PCR-RFLP techniques. The restriction fragments length polymorphisms (RFLP) enabled scientific identification and genetic differentiation between the studied groups. The polymerase chain reaction (PCR) facilitates amplification and analysis of fragments or DNA genes. The selected CYT b gene of each group has been amplified by using PCR technique. RFLP profiles of genes were obtained by digestion with twelve restriction enzymes **(*Apo*I,*Bse*RI,*Pst*I , *Afe*I, *Spe*I, *Eco*RI, *DraI*, *Ase*I, *BanI*, *Hind*III , *Acs*I** and ***Mae*III**). This approach has enabled us to predict the mutation that occurs on "CYT b"gene of the mice embryo of the different studied groups.

Introduction

Phenytoin [(5, 5-diphenylimidazolidine-2, 4-Dione)]. Is a highly effective and widely prescribed anticonvulsant agent which can be useful in the treatment of epilepsy, dermatology and it is one of the most commonly used first-line or adjunctive treatments for cardiac arrhythmias, Lennox-Gastaut syndrome and childhood epileptic syndromes **(Cuskelly *et al*., 2007).**Phenytoin bind to specific site on voltage dependent sodium channels and is thought to exert its anticonvulsant effect by suppressing the sustained repetitive firing of neurons by inhibiting sodium flux through these voltage dependent channels.Phenytoin stabilizes membranes, protecting the sodium pump in the brain and in the heart. It limits the development of maximal convulsive activity and reduces the spread of convulsive activity from a discharging focus without influencing the focus itself

Phenytoin is classed as a teratogen risk factor D. The epileptic pregnant woman takingPhenytoin, either alone or in combination with other anticonvulsants, has a two to three times greater risk of delivering a child with congenital defects. It is not known if this increased risk is due to antiepileptic drugs, the disease itself, genetic factors, or a combination of these. A recognizable pattern of malformations, known as the fetal hydantoin syndrome has been described and includes craniofacial and limb abnormalities, cleft lip and impaired growth defects **(Lin & Lu., 1997 and Camble *et al*., 2006).**

Phenytoin drugs during pregnancy, have adverse affect identified in infants and children of women treated with these medications during pregnancy. Major malformations, mid face and digit hyperplasia, microcephaly, growth

restriction, and deficits in heart are sometimes seen, although the pattern of abnormalities and specific effects vary for individual drugs **(Holmes *et al*., 2001 and Wyszynski *et al*., 2005).**

Phenytoin also significantly depresses interferon augmentation of natural killer (NK) cell cytotoxicity in a dose-dependent manner. Phenytoin suppresses the production of cortisol. Specifically, Phenytoin induces the liver cytochrome P450 enzyme system and stimulates steroid clearance. It can also induce adrenal suppression. Phenytoin can alter vitamin levels. Pellagrous dermatitis has been induced by Phenytoin in children. Phenytoin can affect biotin metabolism It can decrease folic acid levels **(Lewis *et al*., 1995; Moffat &Bernstein, 2007;Kaur *et al*., 2002 and Banhidy *et al*., 2007).**

Phenytoin cause hypertrophy in the liver as proliferation of the endoplasmic reticulum and down regulated the liver tricarboxylate carrier gene which functions to transport citrate and malate from mitochondria. Previous research has shown that Phenytoin altered lipid accumulation in the liver, hepatocyte apoptosis and hyperplasia**.**Phenytoincaused a significant hepatotoxicity including, necrosis, steatosis, DNA damage, hypertrophy, phospholipidosis and hepatic carcinoma in the fetus of rats that received high doses at about half of the gestation period.Phenytoinhypersensitivity syndrome occurs and is characterised by hepatitis. Overall mortality rate when liver is involved is between 18% and 40%**.** The hepatitis is usually anicteric **(Cuskelly *et al*., 2007).**

Phenytoin can cause irreversibly modify critical embryonic or prenatal cellular targets, initiating a deregulation process that can cause in uterus death or teratogenesis. Also controlled activation of mutation in mitochondrial (mtDNA) that can trigger an increase in reactive oxygen species (ROS) level this mutation may cause effect on the component of the electron transport chain **(Robson., 1990; Wells & Kim, 1997 and Lee &Lip., 2003)**

Cytochromes play an active part in the metabolic reactions known as oxidative phosphorylation, by which molecular oxygen is reduced to form water and foodstuffs are oxidized to form carbon dioxide, water, and other oxidized products, resulting in a release of energy **(Alberts., 1994 and Schuetz *et al*., 1994).**

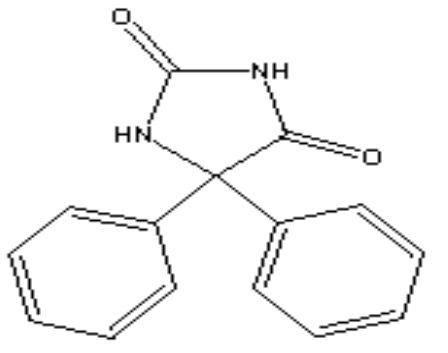
Cytochrome b (CYT b) gene complex is composed of ten proteins subunits in mammalian mitochondria.**CYT b** gene containing a low potential (LP) and a high potential (Hp).**CYT b** gene expressed mainly in liver and gut gravid uterus and placenta. **CYT b** is one of three redox subunits present in both the complex and the cytochrome b6f complex**.CYT b** is a transmembrane lipoprotein - also known as the bc1 complex or ubiquinol-cytochrome c reductase, cytochrome b6, also known as the b6f complex **(Crofts& Berry., 1998; Scheff., 2001 and Stroebel *et al*., 2003).**

MATERIAL AND METHODS

Drug used:

Phenytoin, [(5, 5-diphenylimidazolidine-2, 4-Dione)] has a molecular formula (C15 H11 N2 O2) and it's one of the principle antiepileptic drugs**.**

"The Structure formula of phenytoin"



Phenytoin is offered by local Egyptian pharmacy. It's white, odorless, tasteless, solid-crystalline and solid-powder. The solution is turbid unless

pH is adjusted to 11, 7 and has molecular weight252.3. **Phenytoin** is very slightly soluble in water, slightly soluble in chloroform and ether. Malting point: 295-298°c **(Li et *al*., 2000 and Perea*etal*., 2001).**

Experimental animals:

The present study was carried out on the albino mice that provided from Theodor Bilharzias research institute. The adult males weight range from (24-30gm) and the adult females weight (22 - 28gm). The males and females were kept in good conditions and good diet (milk, water, rice and bread). Adult females of age 2-3 months selected, each four of them were kept with one adult male (in one cage overnight). In the next morning pregnancy was assured by the presence of vaginal plug, and this day was considered to be the first day of pregnancy, assuming that coitus took place at 1.0 a.m. (antemeridian) at the night mating**.** Vaginal plug persists usually for 18-22 hours**.**

Animal Design:

Males and females mice were grouped into four groups each include 20 individuals: **G1**-concidered as the normal males and females. **G2**- Experimental males were treated and allowed to mate with normal females. **G3**-Experimental females were treated and allowed to mate with normal males. **G4**-Experimental males and females were treated and then they were allowed to mate with each other.**G2** –**G4** injected intraperitonealy by **phenytoin (8mg/kg)** for 15 days before mating. Pregnant Females were injected intraperitoneally continuously at the 21th day of gestation. At delivery the fetuses of each group are collected. In this study, the molecular techniques investigate the effect of the drug (in each group) on CYT b gene of mice embryo at delivery by PCR, RFLPs and PCR-RFLP techniques.

Molecular Technique:

DNA Extraction:

(Awwad, 2003 and Gachon *et al*., 2004):

The tissue of early postnatal newborn (at delivery) in each studied group was homogenized in lyses' solution **UNSET** which consists of:

- 0.2 % SDS (20 µl, 10 % SDS)
- 10 mMTris (pH 7.4) (10 µl)
- 10 mMNaCl (2 µl)
- 10 mM EDTA (20 µl, 0.5M)
- 950 µl or up to 1 ml sterile ddH_2O

Then phenol chloroform extraction was used three times to separate the organic and aqueous phases as in the following steps **(Awwad, 2003):**

1- Homogenized tissues are mixed in vortex for 10 sec.
2- Lyses cells in water bath (60 ^oC, 2 hours).
3- Divide the total volume (1000µl) into two 1500µl micro tubes (500 µl).
4- Add equal volume of phenol (500 µl).
5- Mix at vortex.
6- Centrifuge 5 min. at the micro centrifuge.
7- Discard the phenol or put the upper layer in a new micro tube.
8- Add chloroform in equal volume (500 µl).
9- Mix in vortex.
10- Centrifuge (5 min).
11-Use top layer, into new eppendorf caps.
12- Add ethanol (100 % cold) to aqueous layer (500 µl).
13-Freeze to precipitate (1 day or more, -20^oC).
14- Spin down DNA pellet (15 min, 4^oC) and discard ethanol and dry under vacuum for at least 30 min.
15-Add 30 µl sterile ddH_2O to DNA and re-suspend.
16-Use 2 µlof the re-suspended pellet was checked by agarose gel electrophoresis for the presence of DNA ***(cheng et al., 1994).***

Preparation of gel & gene amplification and purification (0.8 % agarose gel):

0.8 g of Agarose (#11388983001, Roche Applied Science, USA) was dissolved in 100ml of 1xTAE buffer (242 g Tris, 3.72 g EDTA, 700 ml H2O, 57.1 ml of glacial acetic acid, the volume brought to 5 litters).

PCR requires several basic components:

-DNA template that contains the region of DNA fragment to be amplified the Cytochrome b gene.

- Primers, which are complementary to the DNA region (CYT b) at the 5´ and 3´ ends of DNA region that is to be amplified to amplify the complete mitochondrial CYT b gene, one µl of whole cell DNA template was used plus oligonucleotide primers**(*Joseph & David, 2001).***

Direction	Primer	Sequence
5´ to 3´	E1M4	TGAGGTGCTACTGTTATTACTA
3´ to 5´	E2M4	ATACGATCATAATTGCTCGATATGG

- *The p*olymerase chain reaction program for amplification of **CYT b** gene was 94ºC 60 sec, 45ºC 2 minutes, 72ºC 3 minutes, 30-35 cycles.
- 1 µl of PCR products was checked by gel electrophoresis for the presence of **CYT b** gene size (~1140bp).
- ***Taq*** DNA polymerase , used to synthesize a DNA copy of the region to be amplified (#M0273L, New England Biolabs, USA).
- Deoxynucleotide triphosphates, (dATP, dGTP, dTTP and dCTP) from which the *taq* DNA polymerase. Used for build the new mitochondrial DNA.
- Buffer solution, which provides a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- PCR products were isolated after separation by agarose gel electrophoresis, using a 3 mmx6 mm 12-well comb. Ethidium bromide was used to stain PCR products in the gel (50mg /100ml 1xTAE) for ten minutes. The PCR products (bands) were visualised under UV lamp and then cut from the gel. Glass milk DNA purification (gene clean kit)(#1001-200, Q Biogene, USA)was used to purify the gene from the agarose gel. Three micro liters of the amplification products were visualized on 0.8% ethidium bromide stained agarose gel to check the quality of amplification. The remaining 7 µl were mixed with 53 µl bidistilled-H2O and divided into 10 µl aliquots for enzyme digestion **(Ram *et al*., 1996).**

Restriction fragment length polymorphism(RFLP)protocol:

To make a digestion in the obtained gene The following reagents were used as follow**:**

Reagent	Volume per sample
Restriction buffer	1.0 µl
Ultrapure water	3.8 µl
BSA	0.1 µl
Enzyme	0.1 µl
PCR product	5.0 µl
Total reaction volume	10 µl

- RFLP profiles were obtained according to the following steps:

1) The water bath was adjusted at temperature 37°C.
2) Combine restriction buffer (1.0 µl), water (3.8 µl), BSA (0.1 µl) and enzyme (0.1 µl), twelve restriction enzymes was used one for tube.
3) Lable 0.5µl tubes.
4) Aliquots of 5 µl of the above-mentioned mixture were added to tube.
5) 5 µl of each PCR product were transferred into labeled tube.
6) The tube was centrifuged briefly then placed in appropriate water bath for 1.5 hours (37°C).
7) The tubes were placed on ice after digestion.

The digestion was performed for ~3.5 hours at ~37°C, and the digestion products were evaluated on 2% TBE-agarose (FMC Bioproducts)gels and stained with ethidium bromide. Bands were detected upon ultraviolet transilluminatation and photographed by digital canon camera (**Rabinow&paul, 1996**).

Restriction enzymes used for CYT b gene RFLPs profiles.

Several restriction enzymes were used in this study; these are**: *Apo*I** (#R0566L)**,*Bse*RI**(#R0581L),***Pst*I** (#R0140L) , ***Afe*I** (#R0652L), ***Spe*I** (#R0133L), ***Eco*RI** (#R0101L),***Dra*I** (#R0129L), ***Ase*I** (#R0526L), ***Ban*I** (#R0118L), ***Hind*III** (#R0104L) , ***Acs*I** (#R0641L) (New England Biolabs,USA), ***Mae*III** (#10822248001)(Roche Applied Science, USA).

Restriction enzymes	Source
Enzyme ***Apo*I**	Isolated from *Arthrobacterprotophormiae.*
Enzyme ***Bse*RI**	Isolated from *Bacillus stearothermophilus* strain.
Enzyme ***Pst*I**	Isolated from *Providenciastuartii* 164.
Enzyme ***Mae*I**	Isolated from *Methanococcusaeolicus.*
Enzyme ***Afa*I**	Isolated from *Acidiphilium facilis* 28H.
Enzyme ***Spe*I**	Isolated from *Sphaerotilusnatans.*
Enzyme ***Eco*RI**	Isolated from *Echerichia coli* RY13,Abgene.
Enzyme ***Dra*I**	Isolated from *Deinococcusradiophilus.*
Enzyme ***Ase*I**	Isolated from *Aquaspirillumserpens.*
Enzyme ***Ban*I**	Isolated from *Bacillus aneurinolyticus.*
Enzyme ***Hind*III**	Isolated from *Haemophilusinfluenzae.*
Enzyme ***Acs*I**	Isolated from *Arthrobactercitreus.*

RESULTS

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique was used to identify the variation of **cytochrome b** (**CYT b**) gene of the four mice groups **(N♂+N♀, T♂+N♀, N♂+T♀, T♂+T♀)**. The resulting restriction bands were separated by agarose gel electrophoresis which helps to differentiate between the four groups collected in photos to identify the different groups.

DNA genome has a high molecular weight, so it remained in the wheel of the agarose gel electrophoresis but the marker DNA (M) was separated into bands with different lengths (Fig. 1). The size of **CYT b** gene product from PCR analysis are approximately about ~1140bp. in all untreated and treated group samples (Fig. 2).

Some restriction enzymes that did not differentiate between the normal and the other three groups **(N♂+N♀, T♂+N♀, N♂+T♀, T♂+T♀); *Apo*I, *Bse*RI , *Pst*I** and ***Mae*III**. The enzyme ***Apo*I** cut the **CYT b** gene of all groups into three bands with lengths (~80, ~270 and ~790bp.) (Table 1and Figs 3 & 15). The restriction enzyme ***Bse*RI** fragmented the same gene of the four groups into four bands, with lengths (~70, ~270. ~370 and ~430bp.) (Table 2 and Figs 4 & 16). The restriction enzyme ***Pst*I** cut the **CYT b** gene; two bands with lengths (~170 and ~970bp.) (Table 3 and Figs 5 &17).However, ***Mae*III** endonuclease cut the studied gene of all groups into three bands with lengths (~190, ~240 and ~710bp.) (Table 4 and Figs 6& 18).

***Afa*I, *Spe*I** and ***Eco*RI** endonucleases divided the four groups into two clusters: **(, N♂+N♀, T♂+N♀** and **N♂+T♀)** and **(T♂+T♀)** (Table 5, 6 & 7 and Figs 7 & 19, 8 & 20 and 9 & 21). **CYT b** gene of **(N♂+N♀, T♂+N♀** and **N♂+T♀)** groups cut with the restriction enzyme ***Afa*I** into three restricted fragments at lengths (~270, ~390 and ~480bp.), however into four fragments in the group **(T♂+T♀)** at lengths (~100, ~190, ~270 and ~480bp.) (Table 5 and Figs 7 & 19). The restriction enzyme ***Spe*I** digested the studied gene of **(N♂+N♀, T♂+N♀** and **N♂+T♀)** groups into four bands (~160, ~240, ~260, and ~480bp.)(Table 6 and Figs 8 & 20). However, ***Spe*I** enzyme restricted the same gene of **(T♂+T♀)** group into three fragments at (~240, ~420 and ~480bp.). The ***Eco*RI** enzyme restricted the **CYT b** gene of **(N♂+N♀, T♂+N♀** and **N♂+T♀)** the studied groups into two fragments (~160 and ~980bp.); whereas fragmented the group **(T♂+T♀)** into three bands (~ 80, ~160 and ~900bp.) (Table 7 and Figs 9& 21).

***Dra*I and *Ase*I** restriction enzymes clustered the four groups into two clusters; **(N♂+N♀** and **T♂+N♀)** and**(N♂+T♀ and T♂+T♀)**.The **CYT b** gene of **(N♂+N♀** and **T♂+N♀)**the studied groups restricted with the ***Dra*I** enzyme into two fragments with lengths (~330 and ~810bp.), but restricted the groups **(N♂+T♀** and **T♂+T♀)** into three fragments with lengths (~150, ~180and ~810bp.) (Table 8 and Figs 10 & 22). The enzyme ***Ase*I** fragmented the **CYT b** gene of **(N♂+N♀,** and **T♂+N♀)**groups into three bands, with lengths (~40, ~190 and ~910bp.), however it restricted the same gene of**(N♂+T♀** and **T♂+T♀)** the studied groups into two fragments with lengths(~130 and ~910bp.) (Table 9 and Figs 11 & 23).

The **CYT b** gene of the studied groups **(N♂+N♀** and **N♂+T♀)** fragmented by ***Ban*I** enzyme into two bands; ~370bp. and ~770bp., and the same enzyme restricted the gene of the group **(T♂+N♀)** into three bands with lengths (~300, ~370 and ~470bp.), whereas the same restriction enzyme gives another three fragments with the group **(T♂+T♀)** at lengths (~150, ~490 and ~500bp.) (Table 10 and Figs 12 & 24).

The ***Hind*III** restriction endonuclease digested the **CYT b** genes of **(N♂+N♀, and T♂+N♀)** groups into two fragments with lengths (~40 and ~1100bp.). However, this enzyme restricted the studied gene of **(N♂+T♀)** group into three bands with lengths (~100, ~250 and ~790bp.), whereas it fragmented the same gene of **(T♂+T♀)** group into four distinct bands with lengths (~40, ~100, ~250 and ~750bp.)(Table 11 and Figs 13 & 25).

The PCR products of **CYT b** gene of the studied groups were differentiated into four clusters when digested with the restriction enzyme
***Acs*I**. The latter endonuclease enzyme cut the **CYT b** gene of the group **(N♂+N♀)**into three bands(~90, ~160 and ~890bp.), the **CYT b** gene of **(T♂+N♀)** group restricted by the same enzyme into two fragments with lengths (~250 and ~890bp.). The ***Acs*I** endonuclease fragmented the **CYT b** gene of **(N♂+T♀)** group into three fragments (~50, ~200 and ~890bp.). Finally, the studied gene of **(T♂+T♀)** group banded into four distinct bands by the same enzyme with lengths (~50, ~200, ~250 and ~ 640bp.)(Table 12 and Figs 14 & 26).

Table (1): Represents the lengths of CYT b genes fragments, which restricted with the endonuclease *Apo*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~80	~270	~790	0
T♂ + N♀	~80	~270	~790	0
N♂+ T♀	~80	~270	~790	0
T♂ + T♀	~80	~270	~790	0

Table (2): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Bse*RI in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~70	~270	~370	~430
T♂ + N♀	~70	~270	~370	~430
N♂+ T♀	~70	~270	~370	~430
T♂ + T♀	~70	~270	~370	~430

Table (3): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Pst*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~170	~970	0	0
T♂ + N♀	~170	~970	0	0
N♂+ T♀	~170	~970	0	0
T♂ + T♀	~170	~970	0	0

Table (4): Represents the lengths of CYT b gene fragments, which restricted with the endonuclease *Mae*III in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~190	~240	~710	0
T♂ + N♀	~190	~240	~710	0
N♂+ T♀	~190	~240	~710	0

T♂ + T♀	~190	~240	~710	0

Table (5): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Afa*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~270	~390	~480	0
T♂ + N♀	~270	~390	~480	0
N♂+ T♀	~270	~390	~480	0
T♂ + T♀	~100	~190	~270	~ 580

Table (6): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Spe*I in the different groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~160	~240	~260	~480
T♂ + N♀	~160	~240	~260	~480
N♂+ T♀	~160	~240	~260	~480
T♂ + T♀	~240	~420	~480	0

Table (7): Represents that the lengths of CYT b genes fragments restricted with the endonuclease *Eco*RI in the different groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~160	~980	0	0
T♂ + N♀	~160	~980	0	0
N♂+ T♀	~160	~980	0	0
T♂ + T♀	~80	~160	~900	0

Table (8): Represents the lengths of CYT b genes fragments, which restricted with the endonuclease *Dra*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~330	~810	0	0
T♂ + N♀	~330	~810	0	0
N♂+ T♀	~150	~180	~810	0
T♂ + T♀	~150	~180	~810	0

Table (9): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Ase*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~40	~190	~910	0
T♂ + N♀	~40	~190	~910	0
N♂+ T♀	~230	~910	0	0
T♂ + T♀	~230	~910	0	0

Table (10): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Ban*I enzyme in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~370	~770	0	0
T♂ + N♀	~300	~370	~470	0
N♂+ T♀	~370	~770	0	0
T♂ + T♀	~150	~490	~500	0

Table (11): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Hind*III in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~40	~1100	0	0
T♂ + N♀	~40	~1100	0	0
N♂+ T♀	~100	~250	~790	0
T♂ + T♀	~40	~100	~250	~750

Table (12): Represents the lengths of CYT b genes fragments restricted with the endonuclease *Acs*I in the studied groups.

GROUPS	Band#1	Band#2	Band#3	Band#4
N♂+N♀	~90	~160	~890	0
T♂ + N♀	~250	~890	0	0

N♂+ T♀	~50	~200	~890	0
T♂ + T♀	~50	~200	~250	~640

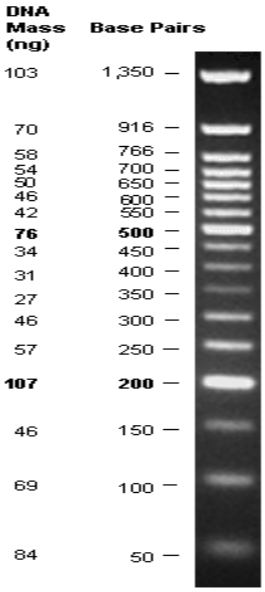


Figure (1): Shows the DNA marker (M) was separated into bands with different lengths.

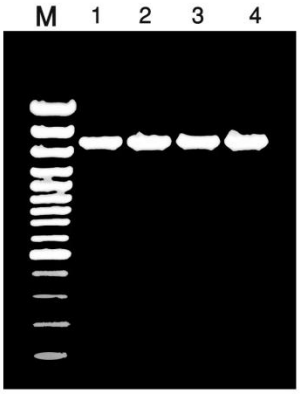


Figure (2): Shows the size of CYT b gene product from PCR analysis is approximately ~1140bp. In normal and treated groups.

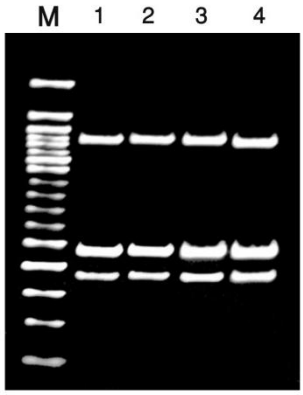


Figure (3): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the bands of PCR products of CYT b gene restricted by the enzyme *Apo* for (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀) groups. In each lane there are three bands with lengths; ~80, ~270 and ~790bp.

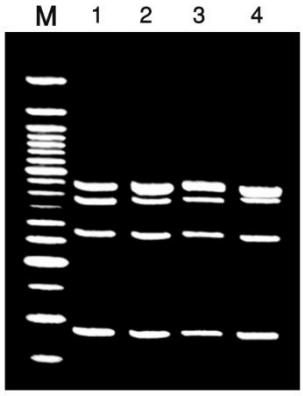


Figure 4): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the fragments of CYT b gene restricted by the enzyme *Bse*RI for (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀). In each lane there are four bands at lengths; ~70, ~270, ~370 and ~430bp.

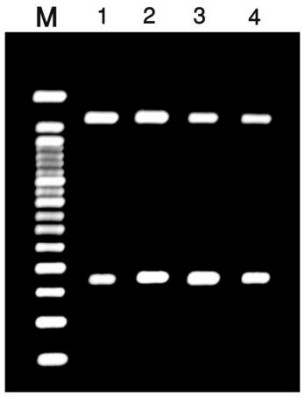


Figure (5): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the restriction fragments of CYT b gene resulted from digestion by the enzyme *Pst*I for the groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀). In each lane there are two bands at lengths; ~170 and ~970bp.

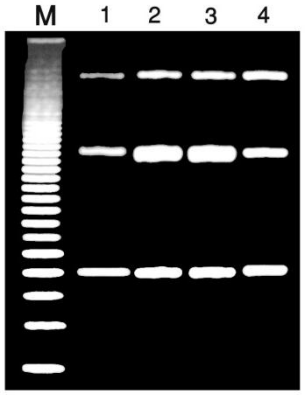


Figure (6): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the restriction fragments of CYT b gene resulted from digestion by the enzyme *Mae*III for the groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀). In each lane there are three fragments with lengths; ~190, ~240 and ~710bp.

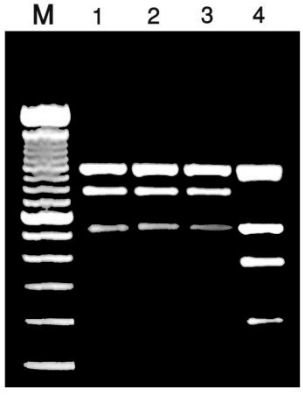


Figure (7): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the fragments of CYT b gene resulted from digestion by the enzyme *Afa*I for the groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀). In each lane there are three restricted fragments at lengths; ~270, ~390 and ~480bp. for the group (T♂+T♀) in lane 4 there are four bands at lengths; ~100, ~190, ~270 and ~480bp.

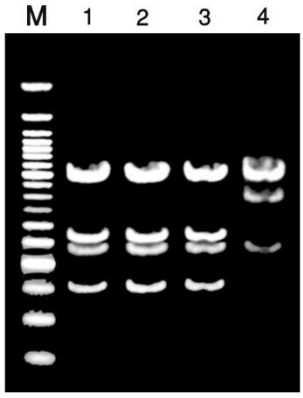


Figure (8): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the fragments of CYT b gene resulted from digestion by the enzyme *Spe*I. From lane 1 to 3 for the groups (N♂+N♀, T♂+N♀ and N♂+T♀) there are four bands with lengths; ~160, ~240, ~260, and ~480bp., whereas the group (T♂+T♀) there are three fragments at lengths; ~240, ~420, and ~ 480bp. In the lane 4.

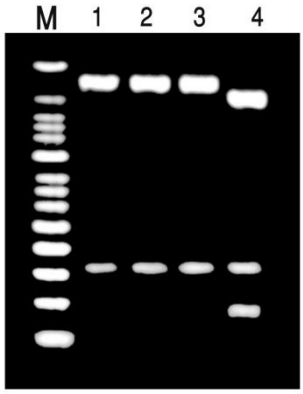


Figure (9): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the bands of CYT b gene restricted by the *Eco*RI enzyme. The groups (N♂+N♀, T♂+N♀ and N♂+T♀) into two bands with lengths; ~160 and ~980bp.from lane 1 to 3. Whereas, the same enzyme cut the CYT b gene of the studied group (T♂+T♀) into three bands with lengths; ; ~80, ~160 and ~900bp.

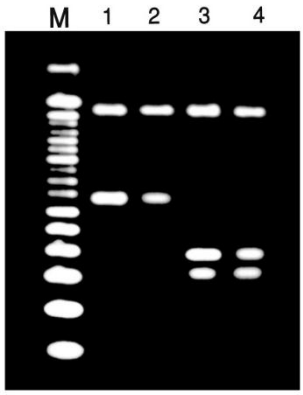


Figure (10): Shows in the first lane the DNA ladder. From lane 1 to 4 represent the bands of CYT b gene restricted by the *Dra*I enzyme. The *Dra*I endonuclease differentiated the four groups of mice into two groups when digested the CYT b gene of (N♂+N♀ and T♂+N♀) groups into two bands with lengths; ~330 and ~810bp. at lane 1&2. But the same enzyme fragmented the gene of (N♂+T♀ and T♂+T♀) groups into three fragments with lengths; ~150, ~180 and ~810bp. at lane 3 and 4.

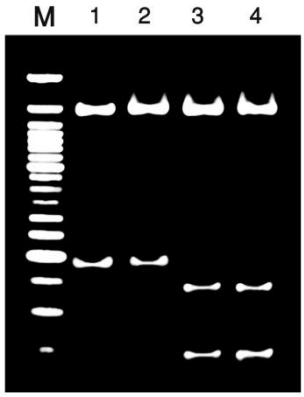


Figure (11): Shows in the first lane the DNA ladder. From lane 1 to 4 represents the bands of CYT b gene restricted by the *Ase*I enzyme. The *Ase*I endonuclease differentiated the four groups of mice into two groups when digested the CYT b gene of (N♂+N♀ and T♂+N♀) groups into three bands with lengths; ~40, ~190and ~910bp. at lane 1&2. But the same enzyme fragmented the gene of (N♂+T♀ and T♂+T♀) groups into two fragments with lengths; ~130 and ~910bp.at lane 3 and 4.

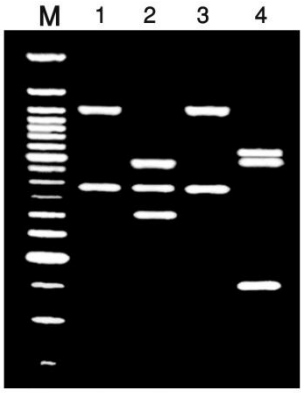


Figure (12): Shows in the first lane the DNA ladder. From lane 1 to 4 represents the bands of CYT b gene restricted by the *Ban*I enzyme. The *Ban*I endonuclease digested the CYT b gene of (N♂+N♀ and N♂+T♀) groups into two bands with lengths; ~370 and ~770bp. at lanes 1&3. But the same enzyme fragmented the gene of (T♂+N♀) into three bands with lengths; ~300, ~370 and ~470bp. at lane 2.Wereas *Ban*I endonuclease cut the gene of (T♂+T♀) group into three fragments with lengths; ~150, ~490 and ~500bp. at lane 4.

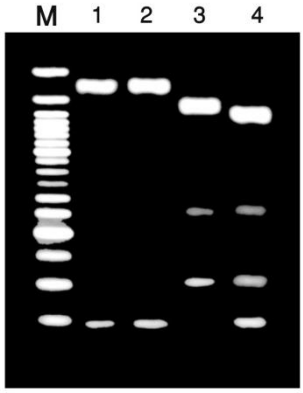


Figure (13): Shows in the first lane the DNA ladder. From lane 1 to 4 represents the bands of CYT b gene restricted by the *Hind*III enzyme. The *Hind*III enzyme digested the CYT b gene of (N♂+N♀ and T♂+N♀) groups into two bands with lengths; ~40 and ~110bp. at lane 1&2. However the same enzyme cut the gene of (N♂+T♀) group into three bands with lengths; ~100, ~250 and ~790bp. at lane 3. And fragmented the gene of (T♂+T♀) group into four bands with lengths; ~40, ~100, ~250 and ~750bp. at lane 4.

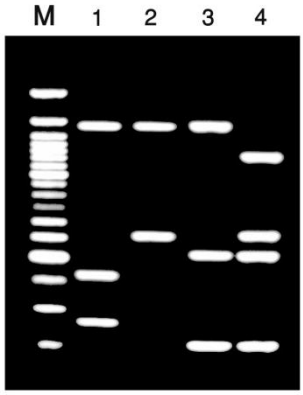


Figure (14): Shows in the first lane the DNA ladder. From lane 1 to 4 represents the bands of CYT b gene restricted by the *Acs*I enzyme. The *Acs*I enzyme differentiated the studied mice groups into four clusters. The *Acs*I restriction enzyme digested the CYT b gene of (N♂+N♀) into three bands with lengths;~90, ~160 and ~890bp. at lane 1.However the same enzyme fragmented the gene of (T♂+N♀) group into two fragments with lengths;~250 and ~890bp. at lane 2. The *Acs*I enzyme cut (N♂+T♀) group into three bands with lengths; ~50, ~200 and ~890bp.at lane 3.And fragmented the CYT b of(T♂+T♀) group into four bands with lengths; ~50, ~200, ~250 and ~640bp. at lane 4.

Restriction fragments

***Apo*I**

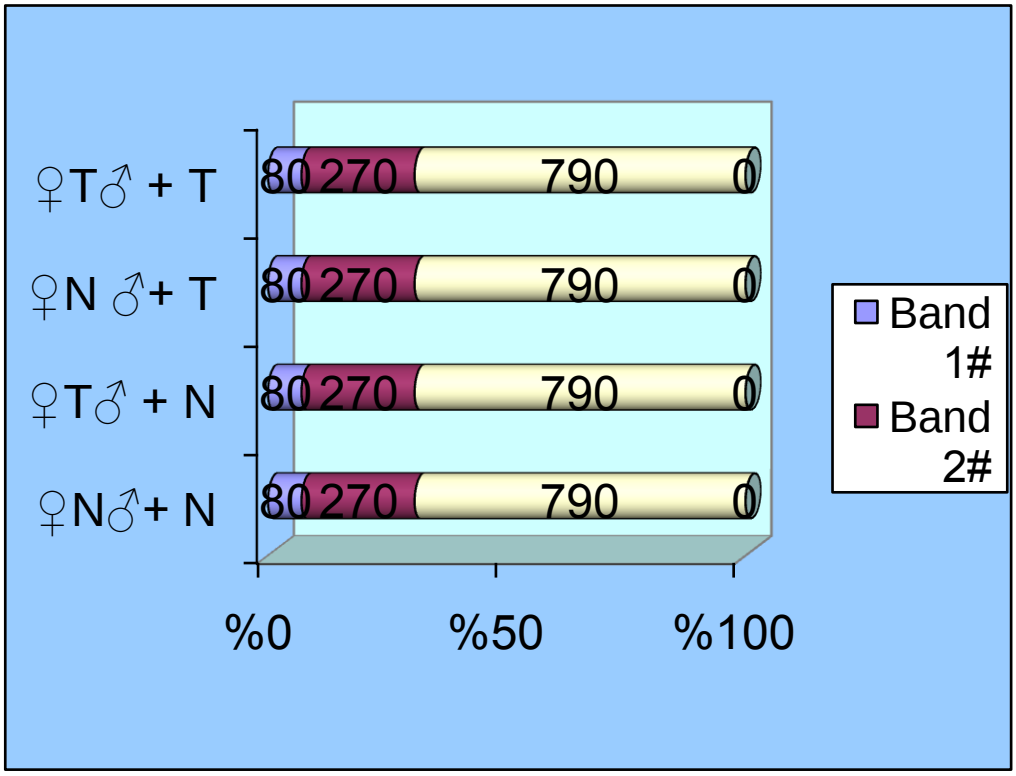


Figure (15): Shows that the *Apo*I enzyme restricted CYT b genes of all groups at (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀) the same lengths; ~80, ~270 and ~790bp.

Restriction fragments
***Bse*RI**

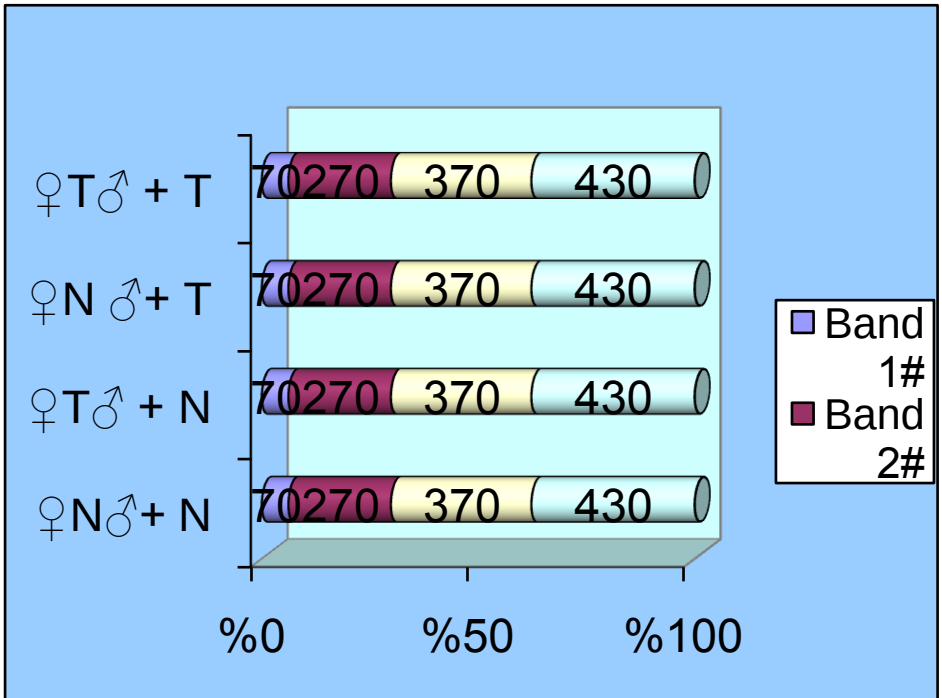


Figure (16): Shows that the *Bse*RI enzyme restricted CYT b genes of all groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀) at the same lengths; ~70, ~270, ~370 and ~430bp.

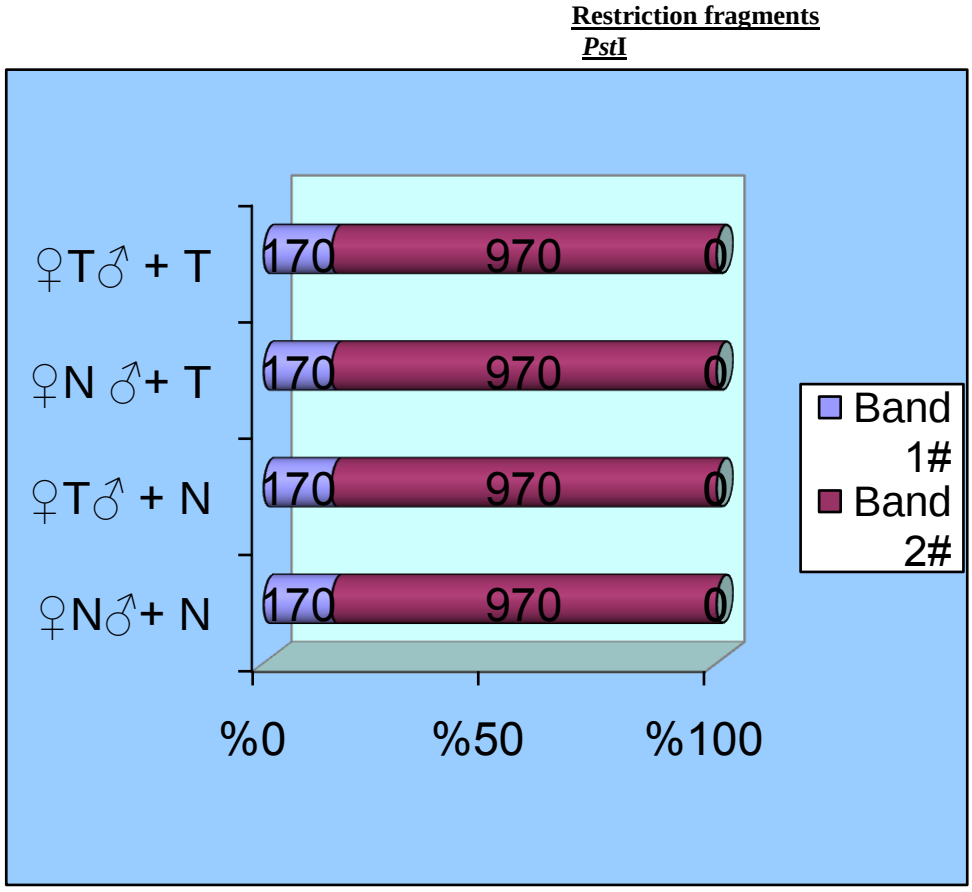


Figure (17): Shows that the *Pst*I enzyme restricted CYT b genes of all groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀). At the same lengths; ~170 and ~970bp.

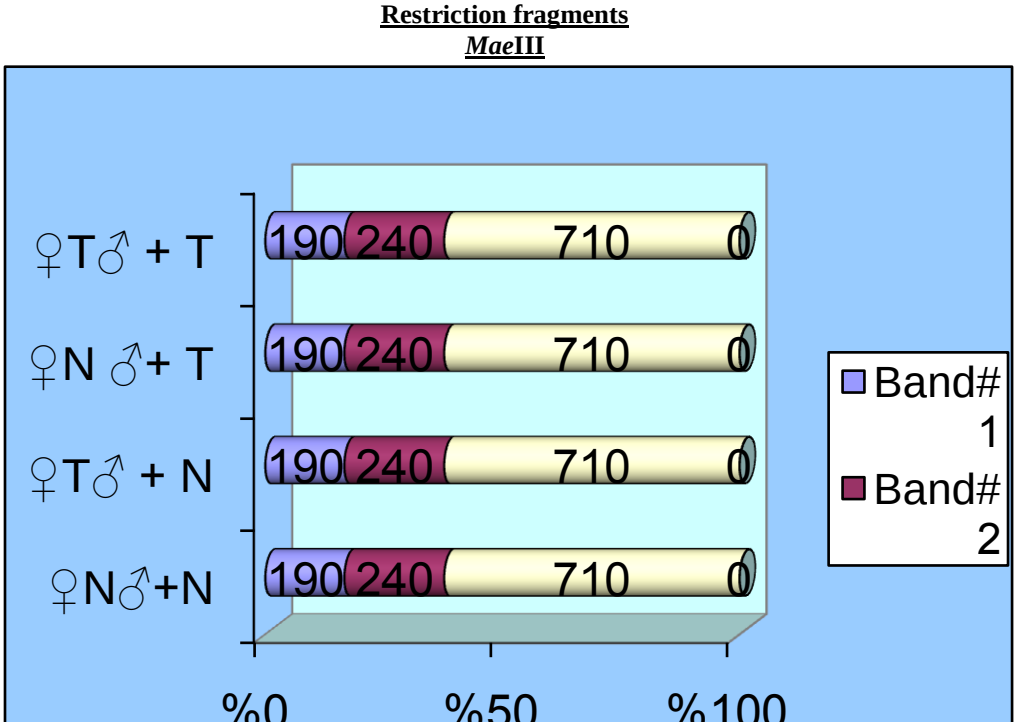


Figure (18): Shows that the *Mae*III enzyme restricted CYT b genes of all groups (N♂+N♀, T♂+N♀, N♂+T♀ and T♂+T♀) at the same lengths; ~190 , ~240 and ~710bp.

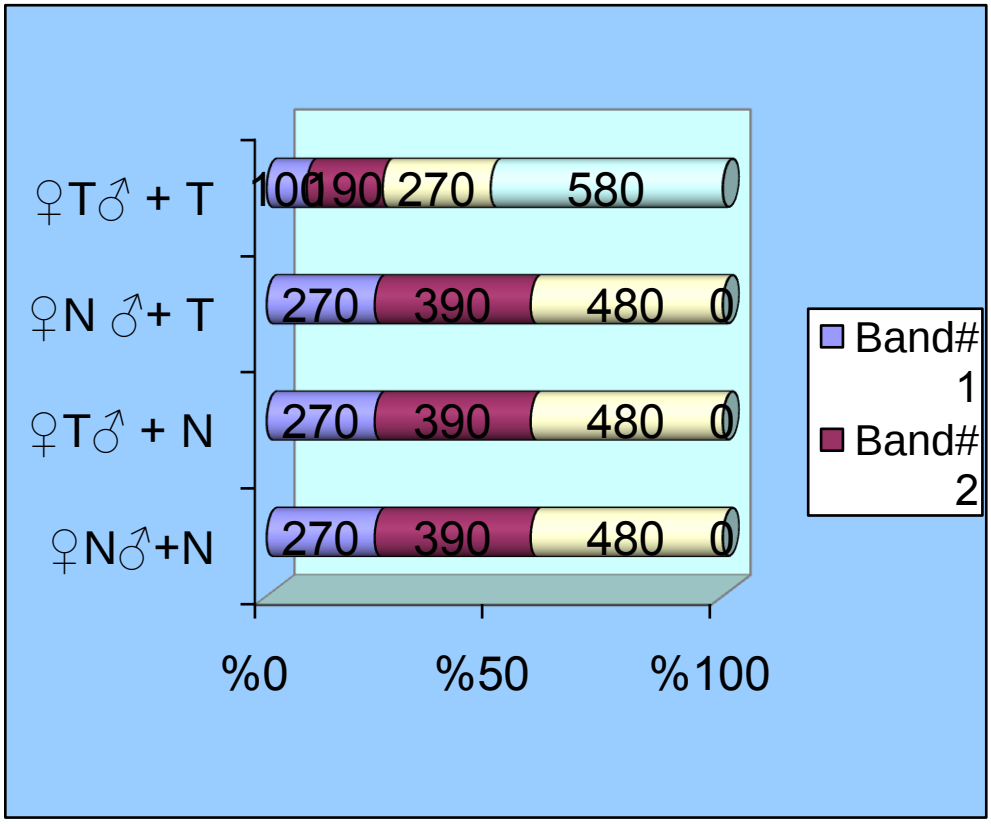


Figure (19): Shows that the *Afa*I enzyme distinguished the group (T♂+T♀) into four bands at lengths; ~100, ~190, ~270 and ~580bp., but restricted CYT b genes of (N♂+N♀, T♂+N♀ and N♂+T♀) into three bands at lengths; ~270, ~390 and ~480bp.

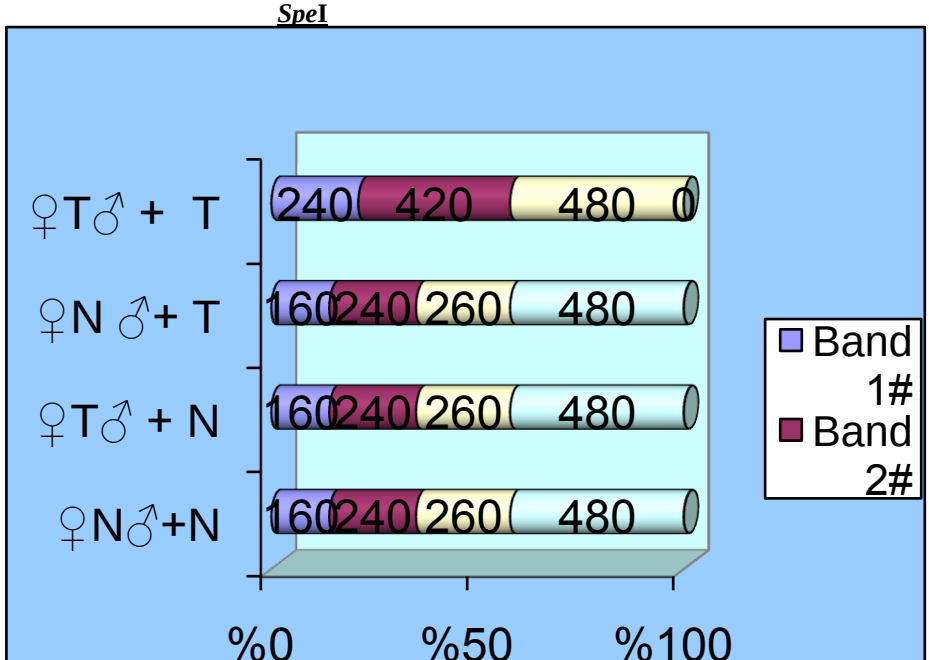


Figure (20): Shows that the enzyme *Spe*I restricted CYT b genes of (N♂+N♀, T♂+N♀ and N♂+T♀) into four bands at lengths; ~160, ~240, ~260 and ~480bp., however there is three fragments with the group (T♂+T♀) at lengths; ~240, ~420 and ~480bp.

Restriction fragments

***Eco*RI**

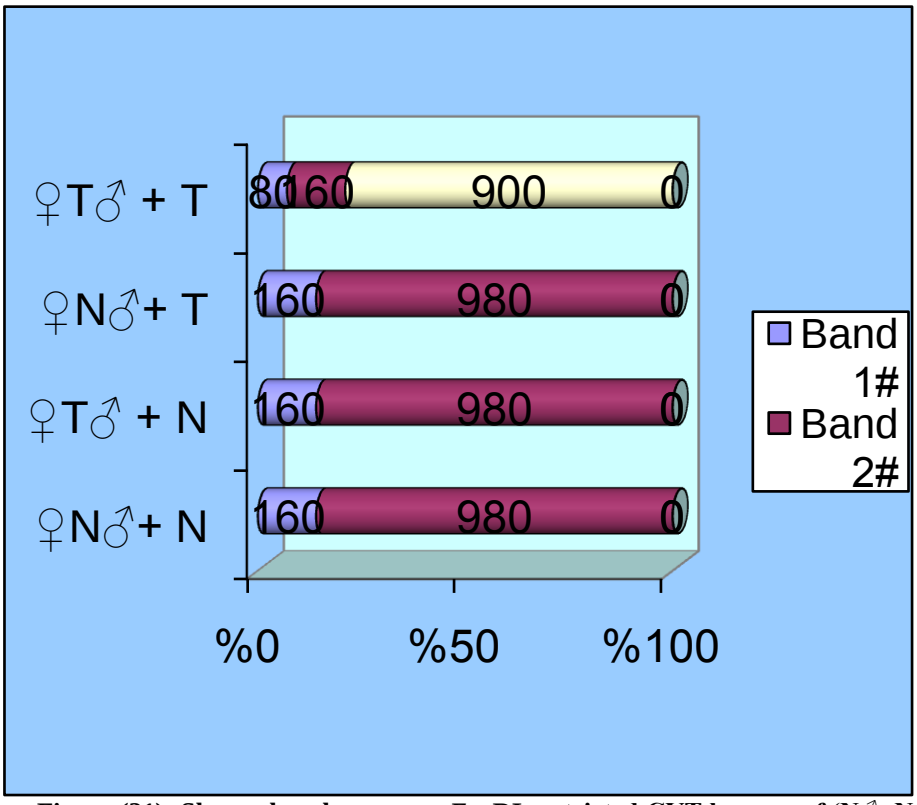


Figure (21): Shows that the enzyme *Eco*RI restricted CYT b genes of (N♂+N♀, T♂+N♀ and N♂+T♀) groups at lengths; ~160 and ~980bp. but produced three bands with the group (T♂+T♀) at lengths; ~80, ~160 and ~900bp.

Restriction fragments

***Dra*I**

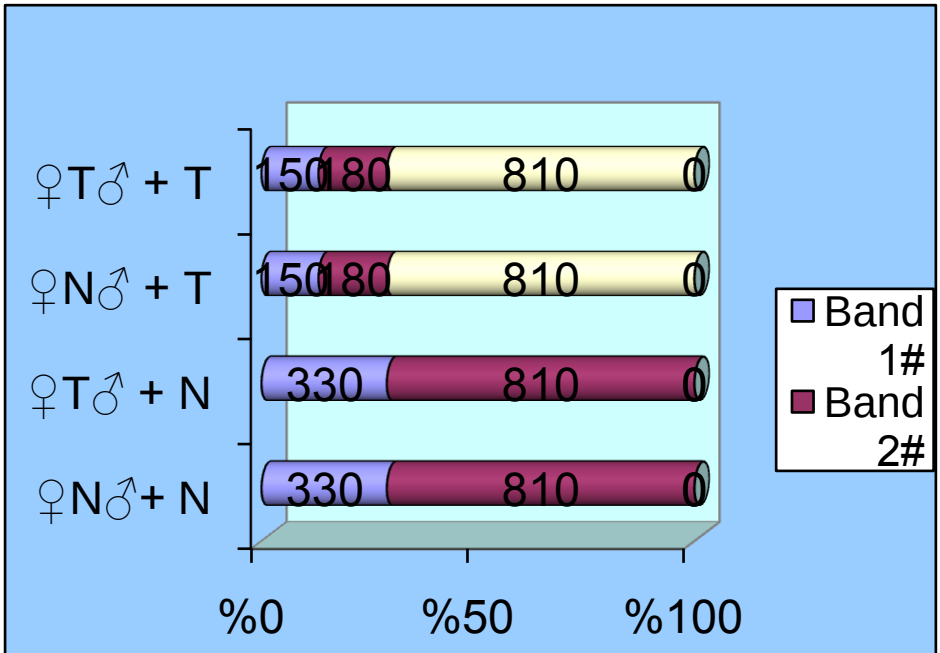


Figure (22): Shows that the restricted enzyme *Dra*I verify the four groups into two separated groups, the CYT b gene of the groups (N♂+N♀ and T♂+N♀) cut with the *DraI* resulted in two bands with lengths; ~330 and ~810bp, but with the other groups (N♂+T♀ and T♂+T♀) there are three bands with lengths; ~150, ~180 and ~810bp.

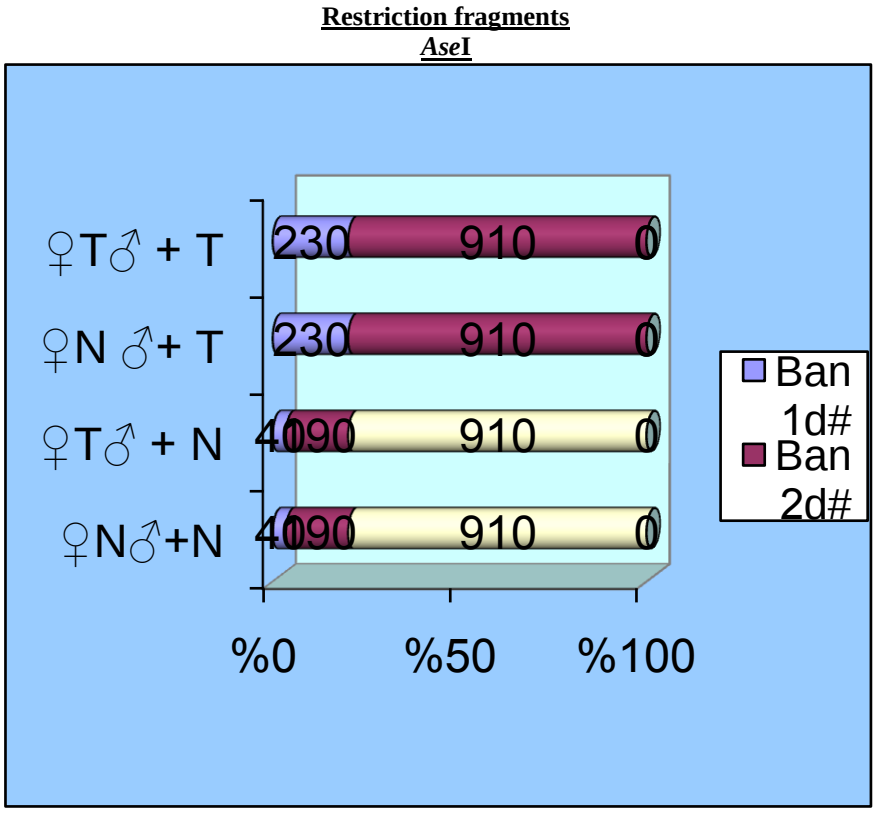


Figure (23): Shows that The enzyme *Ase*I fragmented the CYT b genes of groups (N♂+N♀ and T♂+N♀) into three bands, with lengths; ~40, ~190 and ~910bp.,whereas the same enzyme digested the same gene of other groups (N♂+T♀ and T♂+T♀) into two bands at lengths; ~230 and ~910bp.

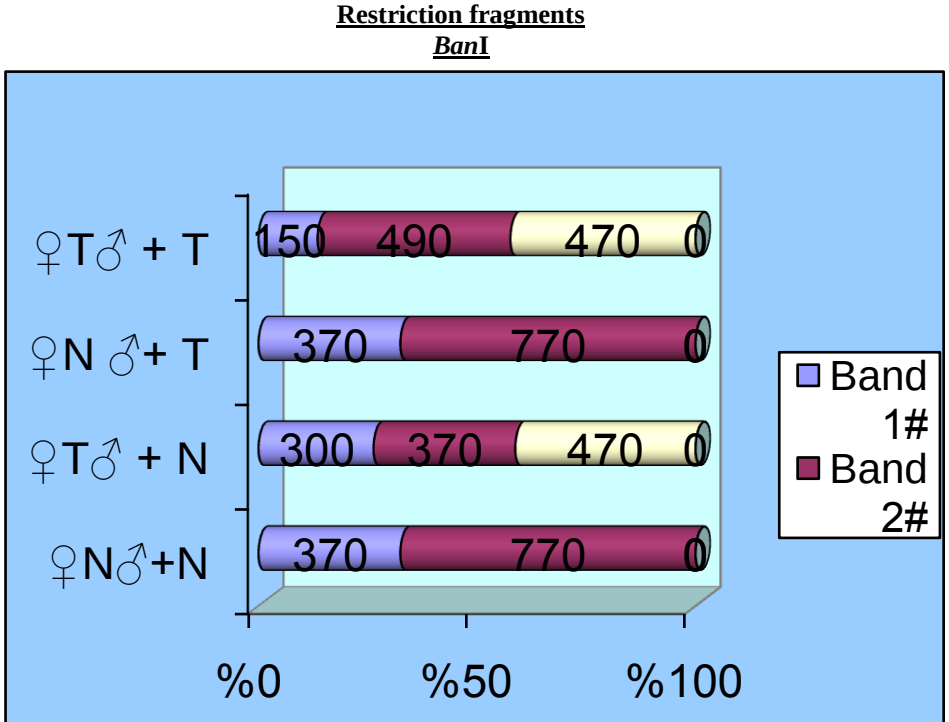


Figure (24): Shows that the *Ban*I enzyme restricted the CYT b gene of the groups (N♂+N♀ and N♂+T♀) with lengths; ~370 and ~770bp. And restricted the gene of the group (T♂+N♀) into three bands at; ~300, ~370 and ~470bp., but produced another three fragments with the group (T♂+T♀) with lengths; ~150, ~490 and ~500bp.

Restriction fragments
***Hind*III**

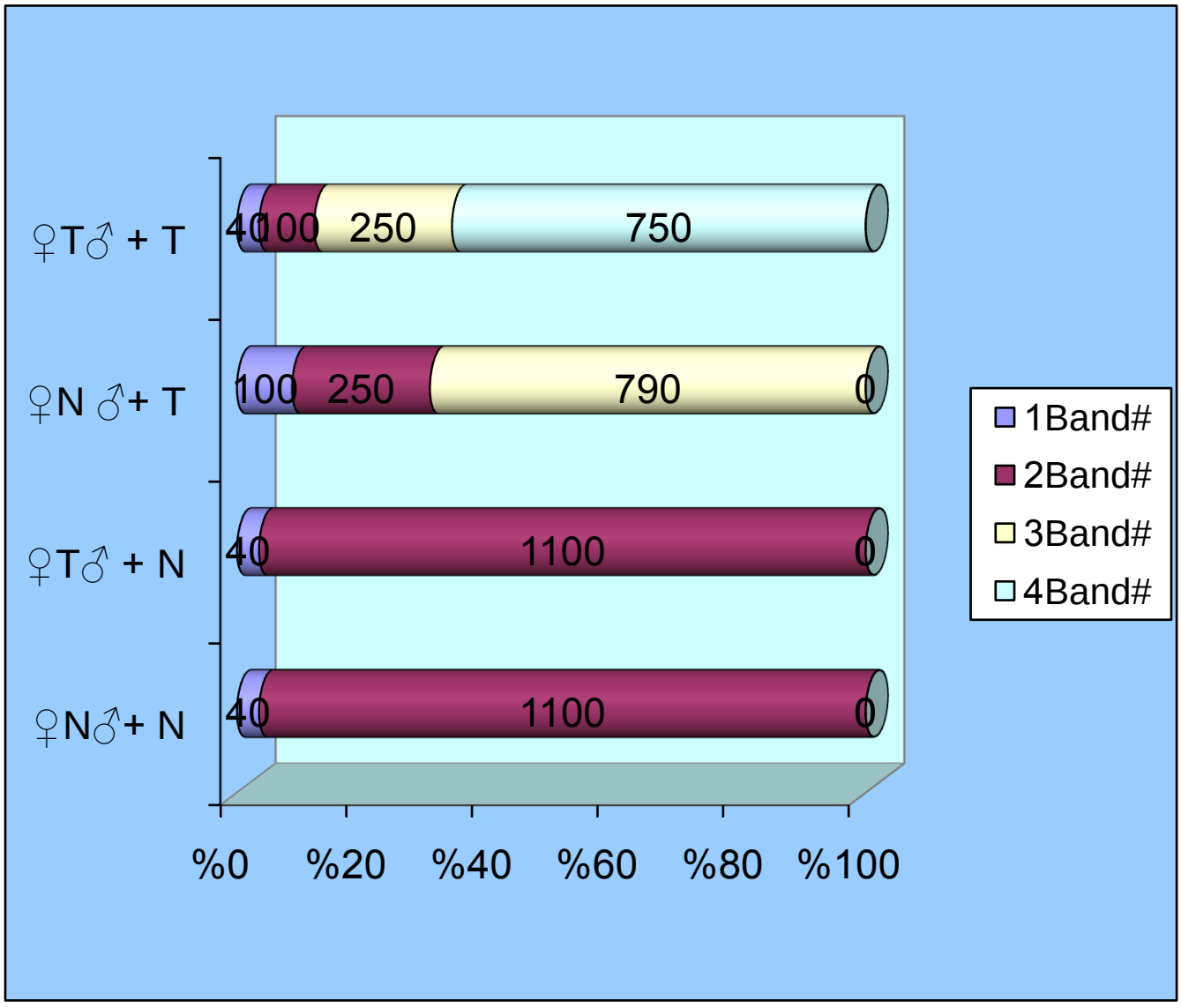


Figure (25): Appears that the enzyme *Hind*III cut the CYT b genes of the groups (N♂+N♀ and T♂+N♀) one cut produced two bands with lengths; ~40 and ~1100bp. In which the same enzyme restricted the CYT b gene of group (N♂+T♀) into three bands at lengths; ~100, ~250 and ~790bp., whereas fragmented the CYT b gene of (T♂+T♀) into four distinct bands with lengths; ~40, ~100, ~250 and ~750bp.

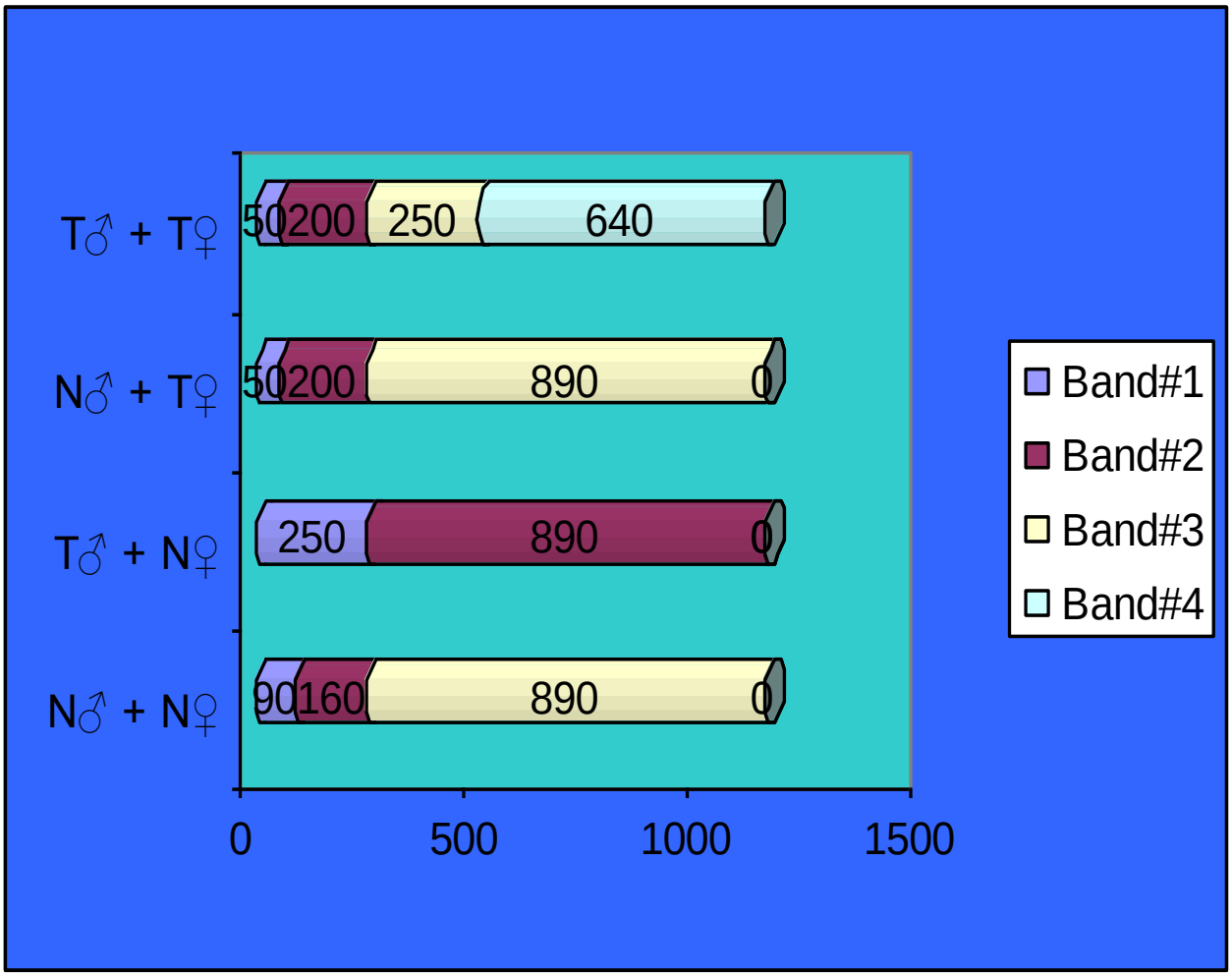


Figure (26): Shows that the enzyme *Acs*I differentiated the four groups, at first the CYT b gene of the group (N♂+N♀) cut twice produced three bands at lengths; ~90, ~160 and ~890bp., the second group (T♂+N♀) restricted by the same enzyme produced two bands with lengths;~250 and ~890bp., the third group (N♂+T♀) fragmented twice produced three fragments with lengths; ~50, ~200 and ~890bp., at last the CYT b genes of the group (T♂+T♀) cut into four distinct bands with lengths; ~50, ~200, ~250, and ~640bp.

Discussion

The present study is concerned with the teratogenic effect of the phenytoin on the early postnatal development (at delivery) of an inbred strain of mice. The dose was applied intraperitoneally for both male and female mice (8mg/kg body weight daily). The results obtained in the present study suggest that phenytoin treatment seems to cause a deleterious effect upon the fetuses of mice. These results are discussed with those arrived at by the previous research workers.

By following the early postnatal viability of the parentally treated mice, it has been found that phenytoin treatment is embryo toxic upon the **cytochrome b (CYT b).**

In the present work,**CYT b** gene was evaluated by PCR-RFLP analysis, making comparison of the molecular weights between the RFLPs of the normal and drug exposed groups, and making molecular embryo

toxicity genotypes according to their **CYT b** gene restriction fragment patterns of (**N♂+N♀, T♂+N♀,N♂+T♀, T♂+T♀**) groups. Based on PCR/RFLP analysis, it was found that the used phenytoin treatment had significantly important for **CYT b** gene mutations. In the present study, twelve type of restriction endonucleases enzymes were used**(*Apo*I, *Bse*RI, *Pst*I, *Mae*III, *Afa*I, *Spe*I, *Eco*RI, *Dra*I, *Ase*I, *Ban*I, *Hind*III and *Acs*I)**to differentiate between untreated and treated groups.

The present study revealed that the restriction enzymes ***Apo*I**, ***Bse*RI**, ***Pst*I** and ***Mae*III** did not differentiate between the four studied groups (**N♂+N♀, T♂+N♀,N♂+T♀** and **T♂+T♀**). Whereas, the restriction enzymes ***Afa*I, *Spe*I and *Eco*RI** detected some mutations in(**T♂+T♀**) group. However, ***Dra*I** and ***Ase*I** restriction enzymes are much more specific enzymes to detect the mutation and the risk of phenytoin treatment of male and female before fertilization.

The present study demonstrated that the endonucleases; ***Ban*I** and ***Hind*III** verify the four groups (**N♂+N♀, T♂+N♀,N♂+T♀** and **T♂+T♀**) into three separate groups. The endonuclease ***Ban*I** restriction enzyme found out the mutation in groups **(T♂+N♀** and **T♂+T♀).** In other word, ***Hind*III** endonucleases detected the mutation of embryo **CYT b** gene when phenytoin treated **(N♂+T♀)** group. As well as, ***Hind*III** enzyme was differentiated the **PHT** mutation on **CYT b** gene embryo **(N♂+T♀** and **T♂+T♀)** groups.

The present data showed that, ***Acs*I** restriction enzyme was detected the gene mutation of the embryos that obtained from**(T♂+N♀,N♂+T♀, T♂+T♀).** Thus, ***Acs*I** restriction enzyme is the most useful enzyme for detecting the mutations of CYT **b** gene of parentally treated newborn. The polymerase chain reaction and restriction fragment length polymorphisms (PCR-RFLP) analysis of the internal transcribed spacer (ITS) region of the DNA has been used for differentiating between the groups of mice**.** PCR-RFLP test used to detect the common mutations in some genes. RFLP analysis could be used in screening for tumors, because it is highly specific, has a low detection limit and is simpler than conventional methods for detecting genetic abnormalities, also useful for detecting codon mutation and allows early proof of molecular determinants of some diseases **(Ram *et al.*, 1996 &Pavlov *et al.*, 2006)** agreed with this**.**

In our study, the detection of polymorphism in **CYT b** gene based on allele-specific PCR methods and PCR followed by restriction fragment length polymorphism analysis (PCR-RFLP) **(Steffensen *et al.*, 2000).** A mutation may create a cleavage site for a restriction enzyme, by this technique mutated alleles by analysis of patterns derived from cleavage of their DNA. If two alleles differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with restriction enzymes. The fragments which differ from the normal fragments will have mutation **(Pennell, 2003)** proved that**.**

In our study, phenytoin could have a mutational effect on the mitochondrial CYT b gene. Molecular analysis of mammalian mtDNA suggest that mtDNA are changing more rapidly than single copy nuclear sequence thus; mutations accumulation mitochondrial DNAs several times faster than in the nucleus. Moreover, mtDNA is susceptible to damage from free oxygen radicals from mistakes that occur during the production of ATP through the electron transport chain. These mistakes can be caused by genetic disorders, cancer and temperature variations. These radicals can damage mtDNA or change them, making it hard to replicate them. Both cases may lead to deletions, rearrangements as **(Boles& Adams., 2005)** mentioned.

In our study adult female mice treated with phenytoin, detected about a 14-fold variation in hepatic **CYT b** protein level, whereas a similar degree of variability was not noted with untreated mice. The investigators suggested that this inter individual variability was due to a genetic polymorphism in their outbreed population of mice. However, the mean hepatic **CYT b** level was still greater in the phenytoin treated groups than in the corresponding untreated group agreed with **(Ghosal *et al.*, 1996).**

The present study emphasized that phenytoin also activates the pregnane X-receptor (PXR) **as (Masuyama *et al*., 2000; Zhang *et al*., 2001&2003 and Wiwi *et al*., 2004).** The PXR regulates the induction of hepatic **CYT b**, which are important enzymes involved in the metabolism of many chemical contaminants and pharmaceuticals **(Sonoda& Evans, 2003).** In contrast, some studies have shown down regulation of **CYT b** protein or **CYT b** activity by phenytoin. Treated female mice demonstrated reduced **CYT b** activity and protein levels **(Acevedo *et al*., 2005).** PXR-null male mice demonstrate greater **CYT b** activity,while PXR-null female mice demonstrate reduced **CYT b** activity compared to untreated mice.Tratologic expression is dependent upon the embryonic intracellular

balance between the processes of bioactivities and macromolecular injury, embryonic detoxification of the xenobiotic reactive intermediate and repair of cellular macromolecules **(Li *et al*., 2000 ;Perea *et al*, 2001 and Von Schacky& Harris, 2007)**mentioned**.**

Generally**,** the present results showed that PCR- RFLP is a simple and rapid technique representing an important advance for studies the effect of the phenytoin drug on the different mice groups, showing variation of the defects between the treated groups in comparison with the control group. The present study documented that the newborn of **(T♂+N♀)** and **(N♂+T♀)** groups, showed lower effect than those **(T♂+T♀)** group.

References

Alberts, B. (1994): Molecular Biology of the Cell. **New. York. Garland. Publishing. Inc., 40-48.**

Awwad, M.H (2003): Molecular identification of Biomphalaria alexandrine and Bulinus truncates using PCR – RFLP of actin gene. **J. Egypt. Acad. Soc. Environ. Develop. 3 (1): 39-52.**

Banhidy, F.; Brian, R. L. and Andrew, E. C. (2007): Risk and Benefit of Drug Use during pregnancy**. Int. J. Med. Sci., 2: 100-106.**

Boles, R.G. and K. Adams (2005): Maternal inheritance in cyclic vomiting syndrome**. Am. J. Med. Genet. A., 133A (1):71-77.**

Camble,K.; Sharova, L. V.; Ward, D. L.; Holladay, S. D. and Neil, E. (2006): Biology: concepts and connections. San Francisco: **Benjamin. Cummings. I. S. B. N., 0-8053: 7160-7165.**

Crofts, A. R. and Berry, E. A. (1998): Structure and function of the cytochrome bc1 complex of mitochondria and photosynthetic bacteria**. Curr.Opin.Struct. Biol., 8: 501-509.**

Cuskelly, W. O.; Hernandez-Diaz, S.; Arbogast, P. G.; Dudley, J. A.; Dyer, S. and Gideon, P. S. (2007): Major congenital malformations after first-trimester exposure to ACE inhibitors. **N. Engl. J. Med. Jun., 8. 354 (23): 2443-2451.**

Gachon , F.; Fabienne, F. O.; Olivier, S.; Patrick, D. and Ueli, S. (2004): Department of molecular biology, national center of competence in research; frontiers in genetics. **Cell Metabolism., 25-36.**

Ghosal, A.; Sadrieh, N.; Reik, L.; Levin, W. and Thomas, P. E. (1996): Induction of the male-specificcytochrome P450 3A2 in female rats by phenytoin. **Arch. Biochem. Biophys., 332: 153-162.**

Holmes, L. B.; Harvey, E. A.; Coull, B. A.; Huntington, B. A.; Khoshbin, S. and Hayes, A. M. (2001): The teratogenicity of anticonvulsant drugs. **N. Engl. J. Med., 344: 1132–1138.**

Kaur, S.; Goraya, J. S.; Thami, G. P. and Kanwar, A. J. (2002): Pellagrous dermatitis induced by phenytoin. **Pediatr.Dermatol., 19: 93.**

Lee, K. W. and Lip, G. Y. H.(2003): The role of omega-3 fatty acids in the secondary prevention of cardiovascular disease. **Q. J. Med., 96: 465-480.**

Lewis, D. P.; Van Dyke, D. C.; Willhite, L. A.; Stumbo, P. J. and Berg, M. J. (1995): Phenytoin-folic acid interaction.**Ann. Pharmacother. Jul. Aug., 29(7-8): 726-735.**

Li, R.; Ciblack, M. A. and Nordoff, N. (2000): In vitro activities of voriconazole, itraconazo leandamphotericin against Blastomycesdermatitidis, Coccidioidesimmitis, and Histoplasmacapsula **Antimicrobial Agents and Chemotherapy. 44(6): 1734-1736.**

Lin, J. H. and Lu, A. Y. (1997): Role of pharmacokinetics and metabolism in drug discovery and development.**Pharmacol. Rev., 49: 403-449.**

Masuyama, H.; Hiramatsu, Y. M. K.; Takafumi, K. and MacDonald, P. N. (2000): Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate pregnane X receptor-mediated transcription**.** ***Mol. Endocrinol.,*** 14**: 421–428.**

Moffatt, D.C.and Bernstein, C.N.(2007): Drug therapy for inflammatory bowel disease in pregnancy and the puerperium. **Best.Pract. Res. Clin. Gastroenterol. 21(5): 835-847.**

Pennell, P. B. (2003): Antiepileptic drug pharmacokinetics during pregnancy and lactation. **Neurol., 61: S., 35-42.**

Perea, S.; Fothergill, A. W. and Sutton, D. (2001): Comparison of in vitro activities of voriconazole and five established antifungal agents against different species of dermatophytes using a broth macrodilution method. **J. Clin. Microbiol., 39(1): 385-388.**

Robson, S. C.; Mutch, E.; Boys, R. J. and Woodhouse, K. W. (1990): Apparent liver blood flow during pregnancy: a serial study using indocyanine green clearance. **Brit. J. Obstet. Gynaecol., 97: 720–724.**

Scheff, I. E. (2001): A century of mitochondrial research: achievements and perspectives. **Mitochondrion, 1 (1):3-31**

Schuetz, J. D.; Beach, D. L. and Guzelian, P. S. (1994): Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. **Pharmacogenetics, 4: 11-20.**

Sonoda, J. and Evans, R. M. (2003): Biological function and mode of action of nuclear xenobiotic receptors.***Pure. Appl. Chem.,*** 75**:1733–1742.**

Steffensen, R.; Thiel, S.; Varming, K.; Jersild, C. and Jensenius, J. C. (2000): Detection of structural gene mutation and promoter polymorphisms in the mannan- binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. **J. Immunol. Meth., 241: 33-42.**

Stroebel,D.;Choquet,Y.;Popot,J.L.andPicot,D.(2003): Ana typical haem in the cytochrome b(6)f complete **Nature,426:413-418.**

Von Schacky, R. and Harris, W. S. (2007): Cardiovascular benefits of omega-3 fatty acids. Cardiovasc. **Res., 73: 310-315.**

Wiwi, C. A.; Gupte, M.; Waxman, D. J.(2004): Sexually dimorphic P450 gene expression in liver-specific hepatocyte nuclear factor 4a-deficient mice. ***Mol. Endocrinol.,*** 18**: 1975–1987.**

Wyszynski, D.; Nambisan, M.; Surve, T.; Alsdorf, R. M.; Smith, C. and Holmes, L. B. (2005): Increased risk of major malformations in offspring exposed to valproate during pregnancy. **Neurology, 64: 961-965.**

Zhang, Q. Y.; Dunbar, D. and Kaminsky, L.S. (2003): Characterization of mouse small intestinal cytochrome P450 expression. ***Drug.Metab.Dispos.*** 31: 1346–1351.

Zhang, Z.; Huang, L.; Shulmeister, V. M.; Chi, Y. I.; Kim, K. K. and Hung, L. W.; Crofts, A. R.; Berry, E. A. and Kim, S. H. (2001): Electron transfer by domain movement in cytochrome bc1**. Nature, 392: 677-684.**