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In Vivo and *In Vitro* Study of the Genetic Effects of Cabergoline Drug

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Abstract

This study aimed to stand on genetic effects important of cabergoline drug. This toxic effect was evaluated for three different doses (0.05, 0.1, 0.5 mg/ml) in comparison with control (PBS/ phosphate buffer saline) both *in vivo* and *in vitro*. *In vivo* study involved the cytogenetic evaluation of cabergoline in mice by examination of mitotic index percentage (MI), micronucleus formation (MN) and chromosomal aberrations. Result indicated that all the tested doses cause significant reduction in MI percentage, while significant rise was seen with both MN formation and all studied chromosomal aberrations. While *in vitro* study involved measuring the effect of cabergoline on normal cell line (REF/ Rat embryonic fibroblast) by studing cell viability through MTT assay and a *TP53* codon 72 polymorphism (rs1042522) through (PCR-RFLP). Results recorded that cabergoline caused high proliferation of normal cells at all doses and p53 polymorphism showed that the Arg allele yielding two fragments 213 and 140 bp after cleaving with *Bstu*I,, while the Pro allele had a single 353 bp band because it did not cleaved by *Bstu*I. In conclusion and according to the results care should be taken while obtaining cabergoline as a results of its genetic side effects.

Key wards: cabergoline, MTT assay**,** PCR-RFLP, polymorphism, Cytogenetic analysis.

دراسه وراثيه داخل وخارج جسم الكائن الحي لتاثيرعقار الكابركولين

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الخلاصه

هدفت الدراسه الحاليه الوقوف على اهم التاثيرات الوراثيه لعقار الكابركولين. وتم تقييم التاثير السمي داخل وخارج جسم الكائن الحي لثلاثه جرع مختلفه (0.05, 0.1, 0.5 mg/ml) بالمقارنه مع السيطرة (دارئ الفوسفات الملحي) . تضمنت الدراسه داخل جسم الكائن الحي تقييم التاثير الوراثي الخلوي من خلال دراسه تقدير نسبه معامل الانقسام الخلوي, تكوين النوى الصغيرة والانحرافات الكروموسوميه . اثبتت النتائج ان جميع الجرعات قد سببت انخفاض معنوي في معامل الانقسام الخلوي في حين سببت زيادة معنويه في تكوين النوى الصغيرة والانحرافات الكروموسوميه. في حين تضمنت الدراسه خارج جسم الكائن الحي قياس تاثير الكابركولين على الخط الخلوي الطبيعي (الارومه اليفيه الجنينيه للجرذ) من خلال دراسه حيويه الخلايا عن طريق فحص MTT وتعدد الاشكال لجين p53 من خلال تقنيه (PCR–RFLP) . اظهرت النتائج ان عقار الكابركولين

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قد سبب ارتفاعاعاليا في معدل انقسام خلايا الخط الخلوي الطبيعي ولجميع الجرع في حين تعدد الاشكال لجين p53 لاليل الارجنين اظهر حزمتين 213 bp و140 bp بعد تقطيعه بـ *BstUI*, بينما اظهر اليل البرولين حزمه واحدة 353 bp ولم يقطع بـ*BstUI* . وعليه وبالاعتماد على النتائج السابقه يجب توخي الحذر اثناء تناول الكالركولين نتيجه لتثيراته الوراثيه الجانبيه.

Introduction

Cabergoline brand names (Dostinex and Cabase) was approved by the FDA on 1996 for treatment of prolactinemia which is characterized by hypersecretion of prolactin which is derived from lactotroph cells in the pituitary gland [1, 2].

However, absorption of cabergoline from the gastrointestinal (GIT) tract is highly different, usually occurring within half to four hours following a single oral dose and since the drug is intended for taken by mouth only, human bioavailability has not been determined [3].

Long-acting dopamine D2 receptor agonist and direct inhibition on the prolactin secretion in the pituitary's lactotroph cells is cabergoline was shown *in vivo* experiments [4]. Although hyperprolactinemia (high levels of prolactin) is treated effectively by cabergoline it can cause side effects symptoms in women who are not breast-feeding or men such as sexual problems, infertility, genetic defects and bone loss [5, 6].

The widely employed indication system for the evaluation of physically, chemically and biologically induced mutations was cytogenetic analysis. The objective evaluation of genetic material damages is allowed by this method that permits direct image analysis for the chromosomal damage. Cytogenetic analyses have been proved to be good and reliable for the mutagen-carcinogen exposure and chromosomal aberration [7].

A number of antiproliferative programs were regulated by p53 gene, including many forms of cell cycle arrest, increased DNA repair, senescence, cell differentiation autophagy, and apoptosis. The cell type (stem cell, differentiated cell or progenitor),, status and stress play a role in regulating the activation of antiproliferative programs [8, 9].

Tumor Protein 53 Inactivation (TP53) tumor-suppressor pathway was considered in many studies [10, 11, 12]. Inactivation has been identified for p53 gene in many mechanisms, such as loss of p53 alleles or many mutations were detected. A polymorphism (rs1042522) at codon 72 in exon 4 encodes either an arginine amino acid (G allele) or a proline (C allele) residue with different biochemical properties [13].

Although cabergoline was known for its activity for treating hyperprolactinemia, but each chemical drug has side effects, and for cabergoline any side effects were seen in the last years and especially genetic effects do not appear at the first time of use and at first few years. Thus present work aims to evaluate the side effect of cabergoline at genetic level through both in *vivo* and *in vitro* study since no previous studies were recoded for genetic effects.

Material and methods

Preparation of Cabergoline: Three different doses of cabergoline (0.05, 0.1, 0.5 mg/ml) were taken and each one was dissolved in 10ml of PBS in separated tubes.

Experimental Animals: In this study twenty-four Albino Swiss BALB/c male mice with an ages between (8-12) weeks and weight ranging between (25-30) gram, were obtained from the Biotechnology Research Center / AL-Nahrain University/ Baghdad/ Iraq, and they were separated into four groups.

Experimental Animals: Four groups of mice were used in this study and divided as follows:

Group I: 6-mice, treated with 0.1ml of PBS. Group 2: 6-mice treated with 0.1ml of 0.05mg/ml dose of cabergoline. Group 3: 6-mice treated with 0.1ml of 0.1mg/ml dose of cabergoline. Group 4: 6-mice treated with 0.1 of 0.5mg/ml dose of cabergoline.

The PBS and cabergoline were given orally for fourteen days, and then the mice were sacrificed at the day fifteenth, and cytogenetic analysis was performed

Cytogenetic Experiments

Mitotic Index (MI) Assay: The procedure was done according to method described by [14] and the calculation of MI percentage was done as follows: under the high power (40X) of the compound light microscope the slides were examined and thousands of non-divided and divided cells were mesured and the rate of MI was counted in the following equation:

$$\text{MI} = \frac{nomber of\ divided cells}{total number of\ the cells\ (1000)} X\ 100$$

Chromosomal Aberrations (CAs) Assay: The procedure was done according to method previously described [14] and the calculation of CAs percentage was estimated as follows: under the oil immersion lens the slides were examined for one hundred divided cells at the metaphase stage of the cell division at which the chromosomal aberrations are clear for each animal.

Micronucleus (MN) Test: The procedure was done according to method described by [14] and the calculation of MN number was done as follows: in thousand counted cells of polychromatic erythrocytes (PCE) the percentage of MN was calculated.

Genotyping Experiment:

Preparation of cell line: The cell line (REF/ rat embryonic fibroblast), is a normal cell line provided by Biotechnology Research Center / AL-Nahrain University/ Baghdad/ Iraq. It was preserved in RPMI-1640 medium supplemented with 10% Fetal bovine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Cytotoxicity assay: this was done according to method by [15]. The cells were seeded in 96-well plates at 10^6 cell/ ml and were treated with cabergoline at three (0.05, 0.1, 0.5) mg/ml doses and PBS was used as negative control. Fifteen µl of dimethyl sulfoxide was added to each well after incubation of the plate at 37 °C for 2hours, and to solubilize the formazan the plates were gentle shaking for 45 min dye. On a microplate reader at 584 nm wavelength the absorbance was determined and the cell growth inhibiting rate was counted.

Genetic polymorphism using (PCR-RFLP)

This experiment was done to confirm the results of cytogenetic study.

Extraction of DNA: After preparation of cell line and treatment with cabergoline from the previous step of cytotoxicity assay (only cell treated with the highest concentration 0.05mg/ml and PBS were examined in this experiment)**,** the cells were harvested after treatment with trypsin, and transferred to a 1.5ml microcentrifuge tube followed by centrifugation at 13,000–16,000 × g for 10 seconds. The cell pellet was washed with PBS after the removing of supernatant once. The genomic DNA was extracted from normal cell line for polymorphism analyses, using a specific Kit (Wizard® Genomic DNA Purification Kit, Promega, USA) according to the manufacturer's instructions.

PCR protocol: P329 was used as the forward primer, and P330 was used as the reverse primer in the *TP53* gene. The procedure for PCR performance was done according to method by [16]. DNA templates were amplified with P329 and P330 primers.

RFLP analysis: The procedure was done according to method by [17] with little modifications.

eight microliters of the PCR product was digested with 2 U of *Bstu*I for at least four hours at 60 °C. the fragments of DNA were run on a agarose gel 2% and the staining was done with ethidium bromide and the fragments sizes were determined by using DNA marker (100 bp).

Statistical Analysis: analysis of one-way variance was used. Data were recorded as mean ± standard error and ANOVA test was used to calculated statistical significances.

Results and Discussion

Rarely focused studies were on the cabergoline dosage effect. Some of these researches focused on correlation of cabergoline with male infertility [18], or either correlation with some serum levels in the body. Other studied concentrate on cabergoline effect on organ tissue destruction [19]. However no previous studies till now studied cabergoline effect on genetic level. The results and data obtained from the present study revealed that cabergoline have obvious cytogenetic effects as shown in table (1and 2). Results in table (1) indicate the effect of cabergoline with three different doses (0.05, 0.1, 0.5 mg/ml) in comparison with PBS as control on mitotic index (MI) and micronucleus formation percentage. Significant decrease in MI as dose increase which reached to (22.11%) at (0.5mg/ml) in comparison with control (51.40%). Also table (1) showed that the effect of Cabergoline on micronucleus formation and results showed that also the effect was significantly dose dependent and high percentage reach to (7.06%) at dose (0.5mg/ml) in comparison with PBS (0.25%).

Table 1-Effect of cabergoline on mitotic index and micronucleus formation percentage in comparison with PBS as control

Groups	**Mitotic Index%** mean ± SE	**Micronucleus%** mean ± SE
Control (PBS) ml	51.40±0.13	0.25±0.11
0.05mg/ml	40.11[a]±0.26	3.10[a]±0.30
0.1mg/ml	31.02[b]±0.63	5.80[a]±0.05
0.5mg/ml	22.11[b]±0.45	7.06[b]±0.51

Moreover, data in Table-2 reflect the effect of cabergoline on different chromosomal aberrations formations which include (ring, gap, acentric, dicentric, chromosome breaks, chromatid breaks, and deletion. Most of these chromosomal aberrations showed significant increase in comparison with control. However, although the underlying mechanisms are still unknown for cabergoline cytotoxicity and genetic aberrations, but one of the suggested mechanism may be the high cell sensitivity to it or the ability of this drug to inter nucleus and interact directly with chromosomes. No previous studies or researches correlated between cabergoline and cytogenetic aberrations in cell.

Table 2-Effect of cabergoline on chromosomal aberrations formation in comparison with PBS as control

Treatment	**Ring (mean ± SE)**	**Gap (mean ± SE)**	**Acentric (mean ± SE)**	**Dicentric (mean ± SE)**	**Chromosome breaks (mean + SE)**	**Chromatid breaks (mean ± SE)**	**Deletion (mean ± SE)**
Control (PBS) ml	0.0 0.0 ±	0.18 ± 1.71	0.0 0.0 ±	0.20 0.6 ±	0.0 + 0.0	0.0 + 0.0	6.0 0.83[c] ±
0.05 mg/ml	0.2 2.2[a b] ±	0.39 0.12[a] ±	1.4 4.23[a] ±	0.50 2.23[b] ±	0.0 + 0.0	0.6 0.42[a] ±	18.4 0.83[b] ±
0.1 mg/ml	0.8 3.2[b] ±	0.59 1.23[c] ±	2.8 0.57[a] ±	0.66 0.02[b] ±	2.0 1.44[b] ±	1.8 0.83[c] ±	28.1 0.83[c] ±
0.5 mg/ml	1.43 7.2[c] ±	0.63 0.89[c] ±	5.8 1.21[b] ±	0.93 0.034[c] ±	3.8 [b]0.860 ±	7.8 0.83[c] ±	35.3 0.83[c] ±

Results in Figure-1, represent the cytotoxicity of cabergoline on normal cell line (REF) at three different doses (0.05, 0.1, 0.5) mg/ml. Results showed that cabergoline increased cell proliferation with all studied doses and the effect was doses dependent and thus high proliferation was seen at high dose (0.5) mg/ml in comparison with control (PBS).

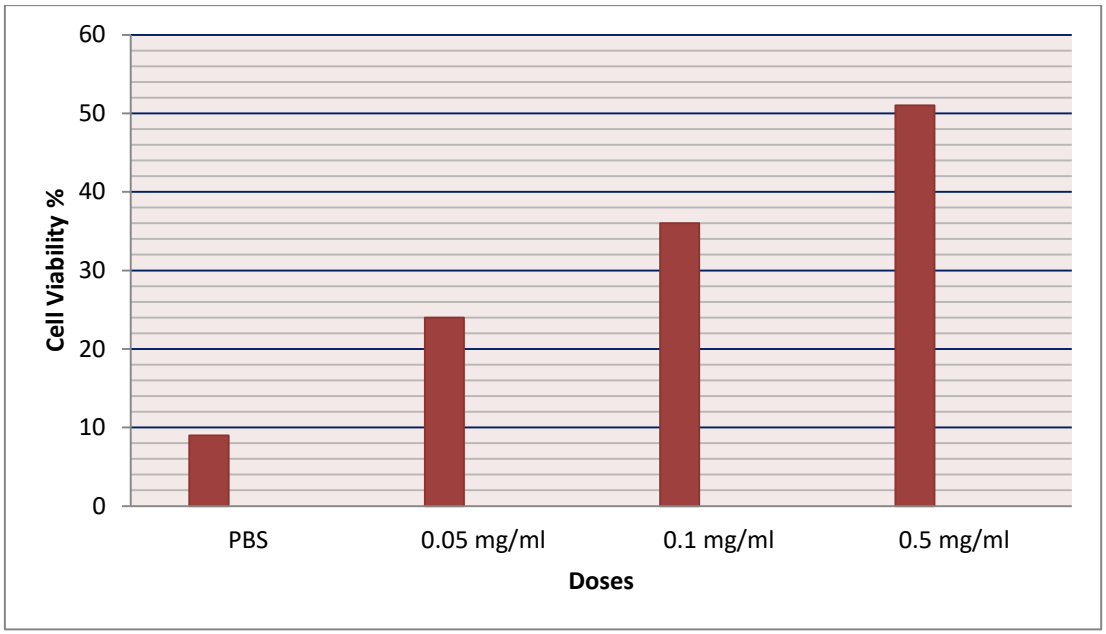

Figure 1-Growth effect of Cabergoline on REF cell line in comparison with PBS as negative control.

Genetic Polymorphism Analyses: The association between *TP53* codon 72 polymorphism and cabergoline drug effect in normal cell line was also investigated in this study. Figure 2 (A and B) showes the analysis of PCR-RFLP product. Figure 2 (A) shows the product of *TP53* with a single band at 353 without drug (PBS negative control), and figure 2 (B) showed the results of *TP53* codon 72 polymorphism in cell line treated with 0.05 mg/ml cabergoline. The Arg allele yielded two fragments 213 and 140 bp after cleaving by *Bstu*I. While, The Pro allele give a single 353 bp band since it was not cleaved by *Bstu*I. Heterozygotes have all the three bands

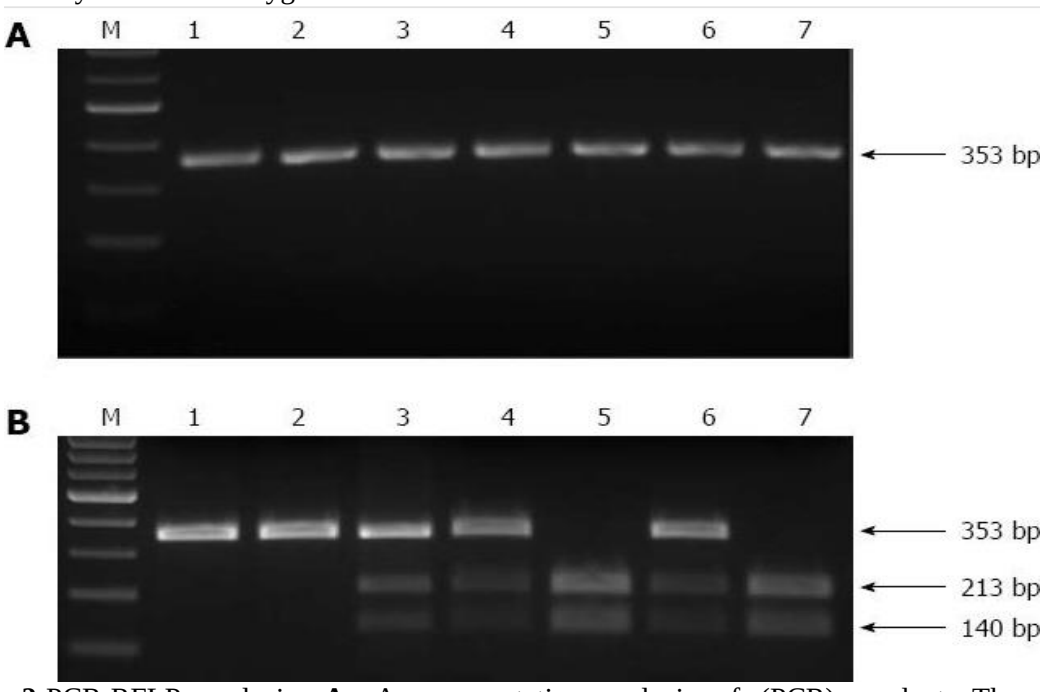

Figure 2-PCR-RFLP analysis. **A:** A representative analysis of (PCR) product. The product corresponded to TP53 with a single band at 353 in normal cell line treated with PBS. **B:** (PCR-RFLP) analysis result of TP53 codon 72 polymorphism. While, the Pro allele give a single 353 bp band since it was not cleaved by *Bstu*I (lanes 1 and 2). The Arg allele yielding two fragments 213 and 140 bp after cleaving by *Bstu*I. (lanes 5 and 7). Heterozygotes contained all three bands (lanes 3, 4 and 6). **M:** 100 bp DNA marker.

In the *TP53* gene more than 200 single nucleotide polymorphisms (SNPs) have been recorded. The codon 72 polymorphism was the most studied one. [20, 21]. The resulting two *TP53* polymorphisms (encoded by codon 72) differ in their biochemical and biological characteristics, such as mobility at electrophoretic [22, 23], localization in cell [24, 25], suppress ability to transformed cell growth and ability to induce apoptosis [26, 27].

However results of genotyping polymorphisms came in concordance with cell viability assay by MTT assay since high proliferation was seen in normal cells after treatment with cabergoline which indicate the occurrence of mutations in one of the genes that correlated with cell cycle proliferation and apoptosis, and in our study p53 gene polymorphism was seen as a result of a mutation that lead to the high proliferation of normal cell. Thus, care should be taken while using cabergoline and especially at long term uses since it may cause increased proliferation of cells or in another sense leading to uncontrolled growth cell cycle due to mutations in one of the ant-apoptotic and cell proliferation genes, p53 , that may lead to cancer development. In conclusion and according to the cytogenetic and genotyping results, care should be taken while obtaining cabergoline for long period as a result of its genetic side effects, and more studies and researchs should be made on cabergoline.

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