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Research Article

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Progesterone Synthetic Hormone (Duphaston) Genotoxicity Assessment by Using Allium Cepa Bio-Assay and RAPD-PCR

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ABSTRACT
Mannny epidemiological studies have established epidemiological studies have established a strong association between maternal hormonal interventions and development abnormalities in progeny like inducing autism behavior. In this study, synthetic progesterone genotoxicity was investigated using the Allium cepa assay. The result of treated cells showed a Clastogenic effect that indicates progesterone interaction with DNA molecules during cell division (chromosome aberrations), such as anaphase bridges, DNA fragments and micronuclei. The results also showed cytotoxic action of the synthetic hormone, such as cytomix, disturbed nuclear membrane, c-metaphase, and early chromosome condensation in prophase. Cell division inhibition at interphase was also observed. The interaction of the widely used synthetic hormone (Duphaston) in Misurata with DNA was documented by RAPD technique using random primers by Using Allium cepa Bio-assay and RAPD-PCR Technique.

Introduction

Monitoring environmental genotoxicity is very important in order to reduce the incidence of genetic disorders. Genotoxicity is defined as a destructive effect on cell genetic material integrity. Genotoxins are substance that interact with genetic material and can be mutagens, carcinogens, or tetragons. Mechanism of genotoxicity depends on genotoxin interaction with DNA: mutagenic, carcinogenic, or epigamic [1,2]. Autism is a lifelong irreversible neurodevelopmental maternal disorder that has a significant impact on families and societies. Many epidemiological studies have established a strong association between prenatal environmental factors and adverse autism outcomes in offspring [2-7].

Little is known about the environmental factors in the etiology of autism. Autism could occur through a combination of factors during the development period. Takumi et al. study suggests that the abnormalities of serotonergic neurons in the brain could lead to synaptic and network alterations in the projecting area relating to social behavior [8]. Progesterone hormone has been shown to modulate neurogenic responses impair the developmental of cognitive responses by down regulating the expression of estrogen receptor β (ERB) and synthetic hormones can bind to receptors and work as natural progesterone [9,10]. The Li, 2018 study shows that the use of progestin at the time of conception, to prevent threatened abortion and prenatal consumption of contaminated sea food during the first 3 months of pregnancy strongly correlated to autism. The mechanism of interaction of environment molecules with DNA during neurodevelopment was of researcher's interest.

Exposure to maternal hormones induces significant ERB promoter methylation and inhibition of ERB expression in offspring [10,11]. The understanding how various risk factors affect neurodevelopment may assist in identifying the etiology of autism early.

Dydrogesterone (Duphaston®) is an orally active, highly selective progestogen that is similar to endogenous progesterone used for many therapeutic purposes and is being used widely in Misurata. When used to preserve pregnancy in women with threatened miscarriage, it is given at a dose of 10 mg twice a

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day [12]. The present study was undertaken with the aim to study the genotoxic effects of different concentrations of this commonly used synthetic progesterone on Allium cepa. Allium assay possesses the ability to interact with the toxicogenic agents during cell cycle and can determine cytogenic and genotoxic effects of harmful chemicals [13-16]. The RAPD-PCR technique can detect DNA molecule damage at molecular level by using different random primers in a PCR reaction. Any changes in pattern of PCR product on gel electrophoresis is evidence of genotoxicity of tested substance [17-19].

Materials and Methods

Allium Cepa Bioassay

Six healthy medium sized onion bulbs with scraped outer brownish scaly skin and dead roots were cultivated in beakers filled with tap water to grow for 4 days at room temperature. four bulbs treated with different concentrations of synthetic progesterone (0.2, 0.1, 0.05. 0.025 mg/ml) for 24 hrs. Two bulbs left as control without treatment. The root tips of treated and untreated bulbs were fixed using Carney's solution (1 glacial acetic acid/3 ethanol ratio) and stored in the refrigerator before staining. Fixed samples were hydrolyzed in 1N HCl at 60Co for 9-15 min. Acetic orcein is used to stain the root tips for 1-2 hrs. The mitotic zones were washed with 45% acetic acid and squashed on a clear slide [20].

Chromosomal aberrations (CA) and nuclear abnormalities (NA) are observed in the different phases of cell division (prophase, metaphase, anaphase and telophase). All cells were photographed using an Olympus microscope connected with camera.

Random amplification of polymorphic DNA (RAPD)-PCR DNA technique

Genomic DNA was extracted from the growing root tips of both (0.025mg/ml) synthetic progesterone treated and untreated onion bulbs. The Quick – DNA™ plant/seed mini prep kit (Zymo Research/ USA) was used for the DNA extraction according to the manufacturer's instructions. The quantity and quality of the extracted DNA were measured at OD 260 and at 260/280 nm absorbance ratios using a NanoDrop Spectrophotometer (Thermo Scientific Company/ USA). Then the extracted genomic DNA was electrophoresed on a 1% agarose gel to check their integrity.

(RAPD)-PCR was performed to generate fingerprints in treated and untreated root tips using 5 random octamer oligonucleotide primers described by Nyati Honegger et al., [21] (Table1). These primers amplified clear and reproducible bands. The polymerase chain reactions were carried out twice using 2x PCR master mix and the reactions were optimized in 20 µl volume containing:10µl master mix, 1µl primer, 2µl Genomic DNA (20ng/µl) and 7µl deionized water.

Table 1: Random octamer primers used in RAPD-PCR.

Primer	Sequence 5' to 3'	Tm (C°)
OPB – 10	GTGATCGCAG	32.00
OPB – 17	AGGGAACGAG	32.00
OPC – 02	GTGAGGCGTC	34.00
OPC – 19	GTTGCCAGCC	34.00
OPD – 20	ACCCGGTCAC	34.00

The reaction mixture was placed in the mini Amppulse thermocycler for 40 cycles/reaction. For Each, including 30s at 94C°, 1min, for primer annealing at 33C°, and 1min for chain elongation at 72C°. The first cycle was proceeded by 5min of denaturation at 95C°, and the last cycle was extended by 5 min elongation step at 72C°.

After the reaction, the PCR products were electrophoresed on a 1.5% agarose gel in in 1x TAE buffer and the DNA band patterns were visualized by GelDoc Go Imaging System. The size of bands was analyzed by comparing their position to the ZR 100bp DNA marker.

Data analysis for RAPD fingerprints

The polymorphism in the RAPD profiles was manually scored and included the appearance of a new band in the treated sample as well as the disappearance of a control band. The RAPD patterns were calculated using the formula $\{1-(a/n)\} \times 100$, where "a" represents the RAPD polymorphic profiles in the root treated with synthetic progesterone and "n" denotes the total number of bands in the control. Changes in the RAPD patterns were expressed as a decrease in GTS [22].

Results and Discussion

The Allium model used to possess the ability to interact with mutagenic agents during its cell cycle and also to determine the cytotoxic and genotoxic effects of harmful chemicals [23]. The chromosomal aberration (CA) test is one of the susceptible approaches for predicting environmental carcinogens or mutagens. The existence of abnormalities in examined cells exposed to a chemical agent indicates that the agent has interacted with the exposed cells' genetic material [1,16,14,24,25].

In this study, abnormal chromosome behavior and nucleus structure were observed in root tip cells treated with the tested synthetic progesterone hormone under a microscope after the appropriate staining procedure. The results in figures 1 and 2 showed the progesterone synthetic hormone induced different types of chromosomal abnormalities in non-dividing (interphase) as well as different mitotic stages. Interphase was the dominant stage in most treated cells (Figure 1). The observed abnormalities were deforming nuclear membrane, cytomix, polyploidy or large nucleus, condensed nuclei and laggard chromosomes which all can be evidences of cytotoxicity of the tested substance. Induced mitosis suppression may be associated with inhibition of DNA and protein synthesis [25,26].

A number of Clastogenic abnormalities were also recorded, such as anaphase bridges and DNA fragments (Figure 2) which indicate that chromosomal aberration occurs due to lesions in both DNA and chromosome spindle protein causing genetic damage [2,16]. DNA fragments and laggard chromosomes can result in micronuclei (MN) observed cells. Micronucleus end point is good effective simple indicator for both cytotoxic and genotoxic action of the tested agents [16,25].

Nuclear abnormality represents an endpoint in chromosomal aberration studies. This condition manifests as morphological changes in the nuclei of cells during interphase. The observed nuclear abnormalities (Figure 1) arise from interactions between synthetic hormones and cellular proteins, leading to

cytotoxic effects in treated cells. These findings demonstrate the genotoxicity of tested synthetic progesterone, consistent with previous studies by Sumedh and Anjankar and Ahmed et al. using human lymphocyte cells [1,24].

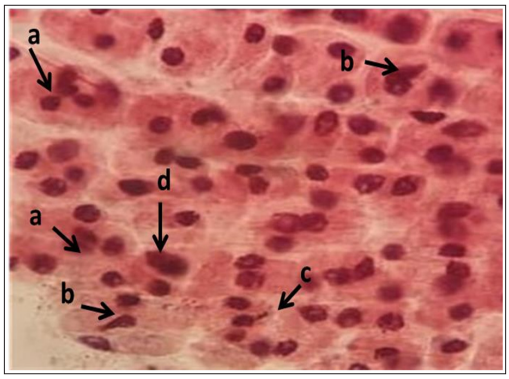

Figure 1: Allium cepa root tip cells treated for 24 hr. with synthetic hormone; a: cytomix, b: abnormal nucleus, c: laggard chromosome or DNA fragment, d: polyploidy. Note: most cell division blocked at interphase.

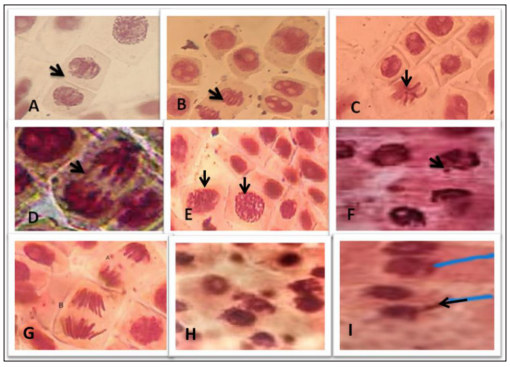

Figure 2: Chromosomal aberrations observed in A. cepa meristematic cells exposed to synthetic hormone for 24 hr. A: normal cell plate formation with incomplete nuclear membrane; B: disturbed telophase; C: c-metaphase; D: anaphase bridge; E: early chromosome condensation at prophase; F: micronucleus; G: Anaphase: A normal, B DNA fragments; H:abnormal nuclear formation; I: laggard chromosome.

RAPD was used to detect the genotoxicity of progesterone at the molecular level using 5 random primers. The product of PCR reaction was analyzed on 1.5% agarose gel electrophoresis (Figure3). Among the primers used, OPD20 exhibits the highest polymorphism, while OPB10 and OPB17 do not reveal any polymorphic band patterns (Table2). As RAPD primers scan almost the whole genome, it can be suggested that OPC02, OPC19 and OPD20 bind the DNA regions in which alterations have occurred. The appearance of new bands such as that generated with OPCO2, OPC19 and OPD20 random primers may be explained on the basis of the mutational events in the genomic DNA of the regulatory system or attributed to the cytological abnormalities observed in treated cells [3].

Additionally, the results of genomic template stability ratios (GTS) were calculated and found to be 57.14% after treatment of A. cepa with (0.025mg/ml) synthetic progesterone. The GTS is a qualitative measure that tracks the alteration in RAPD banding patterns. The GTS is a reflection of the level of DNA damage and efficiency of DNA replication and repair, so a high level of DNA damage that inhibits DNA repair leads to a decreased GTS value. Previous studies have also reported that differences in the RAPD profile may be caused by mutations, chromosomal rearrangements, and other DNA abnormalities; as a result of DNA changes, the primer may lose its annealing site or acquire additional binding sequences [18].

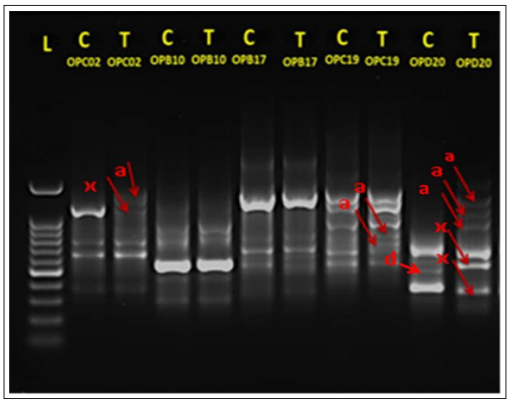

Figure 3: RAPD profile of genomic DNA from A. cepa exposed to (0.025mg/ml) synthetic hormone. C: control samples. T: treated sample. OPC02, OPB10, OPB17, OPC19 and OPD20 are primers. Arrows indicate some of the band changes. a: appearance of new band. d: disappearance of control band. X: change in thickness.

Genotoxicity refers to interaction with or damage to DNA and/or other cellular components that regulate the fidelity of the genome. The exposure of the fatal genome to the synthetic hormone or air pollution during the first 3 months of pregnancy may lead to genome disability by epigenic or mutagenic interaction. The results of this study confirm the genotoxicity of synthetic hormones suggested in other studies by mutagenic action [9,2,24,11]. Therefore, observed chromosome aberration by cytotoxic and Clastogenic action of synthetic hormone, in addition to evidence of changes in RAPD profiles in treated cells that are related to DNA damage, may cause abnormalities in the fetus.

Table 2: Polymorphic bands in RAPD fingerprint upon treatment with (0.025mg/ml) synthetic hormone.

Primer	Control (TB)	After treatment			Polymorphic bands (PB) (a+b+x)
		a	b	x	
OPB – 10	5	0	0	0	0
OPB – 17	4	0	0	0	0
OPC – 02	4	1	0	1	2
OPC – 19	5	2	0	0	2
OPD – 20	3	3	1	2	5
	21				9

TB: total band. PB: polymorphic band, a: appearance of new band. d: disappearance of control band. X: change in thickness.

Conclusion

The findings made from the present study demonstrate the possible genotoxic consequences of synthetic progesterone hormone (Duphaston). Duphaston may cause DNA damage and genomic instability, which could raise the risk of mutations and other genetic abnormalities in the fetus. This is supported by the chromosomal aberrations in Allium cepa that have been identified as well as the variations in DNA banding patterns found by RAPD-PCR. The long-term effects of Duphaston exposure should be further studied, and a new attitude about widespread usage of hormonal steroids these days is required to be changed.

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Ethics

There are no ethical issues that may arise after the publication of this manuscript.

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