

## GENOTOXICEFFECT OF CONTRACEPTIC PILLS

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(Received on Date: 4<sup>th</sup> October 2016

Date of Acceptance: 15<sup>th</sup> November 2016)

### ABSTRACT

In present study, the ability of two types of contraceptive pills (Microgynon ED Fe) on DNA fragmentation in human lymphocytes was studied, where these cells represent important defense line of the body. White coated tablet of contraceptive pills at high concentrations (1000 µg/ml) were achieved a significant fragmentation ( $p \leq 0.05$ ) on lymphocytes DNA at different exposure period (24hr, 48hr, 72hr); percentages of DNA fragmentation was 66.8%, 68.7%, 66.6% respectively. On the other hand, Red coat tablet at same concentration had highly apoptotic activity against normal lymphocytes; the percentage of fragmented DNA was (68.6%, 65.9%, 67.2%) respectively at (24hr, 48hr, 72hr) exposure time. there was significant differences ( $p \geq 0.05$ ) when compared with untreated cells, where present results showed low percentage of DNA fragmentation (21.14%, 21.78%, and 23.42%) in untreated cells after different exposure periods (24, 48, and 72hrs) respectively. As well as, the present studies were conducted to determine toxicity of drug in rabbit's in order to obtain scientific information about its safety. Toxicity of contraceptive pills at (1000 µg/ml per day) for two weeks was investigated, histopathological changes for (heart, liver, ovary, intestine, lung, and kidney). Rabbits treated with drug showed pathologic changes in the intestine include epithelial erosion, PMN infiltration referring to acute inflammation, as well as, distortion shape. On the other hand, liver sections presented with high-grade dysplasia (pre-cancerous lesion), highly infiltration of RBCs (blood lysis), also section exhibit fatty degeneration. The distribution of contraceptive pills in the various tissues namely, lung and heart upon oral administration of contraceptive pills to rabbits group showed blood lysis, distortion of shape, air vacuoles formation, in lung tissues. Recently, heart section revealed highly blood lysis.

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**No:of Tables 1**

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**No: of References : 42**

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## INTRODUCTION

Oral contraceptives are the combination of estrogen and progesterone. They are used to treat various hormonal disturbances, premenstrual syndrome and ovarian cysts [1 Siddique and Afzal, 2005]. The composition of pills varies from country to country due to the responsiveness variation from individual to individual or one population to another [2]. Synthetic progestins and estrogens have been reported to be toxic in various experimental models. Their prolonged use has been reported to induce cancer in humans. [11], studied the effects of oral contraceptives among users using chromosomal aberrations, sister chromatid exchanges and DNA damage as a parameter, in cultured human peripheral blood lymphocytes [1]. The conversion of estrogen into catechol estrogens and quinines, via redox reactions causes oxidative damage to DNA [5,7]. There are both positive as well as negative reports regarding the genotoxic effects of estrogens and synthetic progestins [8–9].

Also they showed that the prolonged and extensive use of these drugs in our daily life may be hazardous and also, that OC users should be aware of multifactorial risk factors (environmental, genetic and life style patterns) that may be responsible for additional DNA damage. Oral contraceptives (OC) by women caused DNA damage in the number of lymphocytes confirmed by measuring the frequency of sister chromatid exchanges (SCEs) and on the response in the alkaline comet assay (single-cell gel electrophoresis

(SCGE)) compared with their age-matched untreated controls [10]. Earlier reports suggest Several studies revealed the genotoxic effects of synthetic progestins in cultured human peripheral blood lymphocytes [3–4]. In these studies the genotoxic effects were observed at very high doses of OC and their effects employed in the reactive oxygen species (ROS) that was suggested as a causative agent for the genotoxic damage [5,6] furthermore significant increase was observed in sister chromatid exchanges (SCEs) Cell among users. In the present study an attempt has been made to investigate the possible genotoxic effects of contraceptives pills in cultured human peripheral blood lymphocytes by using DNA damage, as well as, the mutagenic effect of oral contraceptives in rabbits (female) tissue as a parameters.

### Material and methods.

#### Cytogenetic study

#### Solutions, buffers and stains for blood lymphocytes culture:

Phosphate buffer saline (PBS) it was prepared according to (29)

Sodium bicarbonate solution it was prepared according to (4.4%)(30)

fetal calf serum (FCS) it was prepared according to (31)

Antibiotic solutions: it was prepared according to (32)

**Colcimied suspension:** It was prepared by dissolving (10) mg of colcimied, in 10 ml of phosphate buffer saline taking into account the preparation instantly when you use and away from light, used in the cytogenetic study to stop cell division in the tropical phase (Metaphase).

**Hypotonic Solutio:** It was prepared by dissolving 0.75 g of potassium chloride (KCl) in 100 ml of distilled water and then infertility by autoclave and store at 4°C until used.

RPMI-1640 (Rosalw Park Memorial Institute) medium it was prepared as (33)

**Maintenance Medium (Serum Free Medium SFM)** it was prepared as (34).

Different concentrations of contraceptive pills (Microgynon ED Fe) were prepared at concentrations (500, 250 and 125, µg/ml) with serum free media.

**Solutions and buffers used for detection DNA fragmentation it was prepared as (35).**

**TE buffer, PH 7.4:** This solution was prepared to be consisting of 10 mM of Tris HCL with 1mM of EDTA in DW. And the pH was adjusted to 7.4, and then the volume of solution was completed to liter with DW.and sterilized in autoclave, then kept at 4°C until use.

**TTE solution:** This solution was consisted of TE buffer and Triton-X100 with concentration ratio of 0.2%

**Trichloroacetic acid (TCA) solution (25%):**It was prepared by dissolving 25gm of TCA in 100 ml of sterilized DW .then stored at 4°C until use.

**Trichloroacetic acid (TCA) solution(5%):** It was prepared by dissolving 5gm of TCA in 100 ml of sterilized DW, and then stored at 4°C until use.

**Diphenylamine reagent (DPA):** This reagent was prepared by dissolving 1.5 gm of DPA in 100ml of glacial acetic acid with using of magnetic stirrer. Then 1.5ml of sulfuric acid was added, a volume of 0.1ml of acetaldehyde was added to 20 ml of this mixture.

### Collection of Blood samples

Peripheral blood (5 ml) was aspirated from each subject. Blood samples were immediately transferred to sterile heprenizedvacutainer tubes for lymphocyte separation.

### Separation of lymphocyte from blood samples as (36)

**Detection ofContraceptive pills effect on the viability of lymphocytes isolated from healthy individuals as (37)**

**Detection of contraceptive pills apoptosis on lymphocytes using Quantitative method by DPA reagents:** Method of Boyum (1968) was dependent.

**Experimental animal:** Four young Iraq White rabbits (weight, 2.5 to 3.0 kg) were used in this experiment, they were fed stock cabbage, carrot as well as, water was supplied *ad libitum*.

**Animal treatments:** Animals were divided equally into two groups, group A represented by three animals, orally treated with contraceptive pills (three

dosage at each day) for 21 days, while group B (negative control group without treatment) received water and diet without drug.

**Histopathological studies:** All animals were anesthetized by xylazine and ketamine hydrochloride, after that killing all groups. All organs collected from animals groups and then transported to glass containers containing (10% formaldehyde), each container labeled with the number and give specific symbols for each sample. After those samples were transported to the histopathological lab for evaluate structural alterations in the tissues after treated with drug, all tissue sections stained by Haematoxylin and Eosin stain.

**Results and Discussion**

**Cytotoxic study.**

In present study, the ability of two types of contraceptive pills(Microgynon ED Fe) on DNA fragmentation in human lymphocytes was studied, First beige coated tablet of contraceptive pills at high concentrations (1000 µg/ml) were

achieved a significant fragmentation ( $p \leq 0.05$ ) on lymphocytes DNA; percentages of DNA fragmentation was 67.2%, after 24hrs of incubation period. On the other hand, present results showed low percentage of DNA fragmentation (21.14%) in untreated cells after exposure periods (24,hrs) (table 1).

Current results showed, that the contraceptive pills second type (brown coat tablet) at high concentration had apoptotic activity against normal lymphocytes after 24hrs of exposure time; the percentage of fragmented DNA was 66.866%, and there was significant differences ( $p \geq 0.05$ ) when compared with untreated cells, where present results showed low percentage of DNA fragmentation (21.14%, 21.78%, and 23.42%) in untreated cells after different exposure periods (24, 48, and 72hrs) respectively.

(hrs) Time	Concentration (1000µg/ml)		
	0	R	W
24	21.14 ± 1.45	68.6 ± 1.75	66.8 ± 2.27
48	21.78 ± 1.62	65.9 ± 2.86	68.7 ± 3.51
72	23.42 ± 1.77	67.2 ± 3.19	66.6 ± 2.89

From the results above, it can be noticed that the toxic effect of contraceptive pills is time-dependent. In addition, the results

showed significant difference between DNA fragmentation percentages of treated and untreated cells with

contraceptive pills during different periods of incubation. However, current results concluded that contraceptive pills drug had highly toxic effect on the blood cells (lymphocytes) and that reflected on all the body activity because these cells ( $p < 0.001$ ), when it compared with untreated cells.

Previous study by (1) it has been speculated that two contraceptive pills components generate active oxygen species causing DNA damage which leads to the carcinogenesis (2). Oral contraceptives are the combination of estrogen and progesterone, the conversion of estrogen into catechol estrogens and quinines, via redox reactions causes oxidative damage to DNA.

The frequency of cells with aberration in OC users was almost similar to their age matched controls. Gaps and breaks of chromosome as well as chromatid were observed [38]. Earlier studies performed on the genotoxic potential of steroids have shown to cause chromosomal damage, induction of SCEs and formation of endogenous adducts [39,40]. Estrogen such as ethinylestradiol, in the liver undergoes aromatic hydroxylation and the product, 4-hydroxyestrone, 3,4-dihydroxy 1,3,5 (10)-oestratrien-17-one (4-OHE) is carcinogenic in male Syrian golden hamster kidney tumor model [41].

Positive associations between contraceptive pills use and the development of the cancer in urinary tract also have been reported. As well as, In a similar study, their results were modestly supportive of the hypothesis that exposure to contraceptive pills, particularly dark contraceptive pills, is a risk factor for

represent important defense line of the body. However, this study shed the light on toxic activity of contraceptive pills on lymphocyte isolated from healthy patients. Contraceptive pills has powerful cytotoxic effects against lymphocyte myeloid leukemia and refractory anemia with excess of blasts (40). In a multicenter case control study on risk factors for acute leukemia and preleukemia, a moderate leukemogenic effect of contraceptive pills use was suggested (41).

Evidence suggests that the genotoxic mechanism of estrogens and its metabolites in breast cancer involves their oxidation to 3,4-quinines and reaction with DNA to form depurinating N3Ade and N7Gua adducts. Authors of the study [42].

The authors of the study [43] examine formation of DNA adducts by reactive electrophilic estrogen metabolites, formation of reactive oxygen species by estrogens and the resulting indirect DNA damage by these oxidants, and, finally, genomic and gene mutations induced by estrogens. Several types of indirect DNA damage are caused by estrogen-induced oxidants, such as oxidized DNA bases, DNA strand breakage, and adduct formation by reactive aldehydes derived from lipid hydroperoxides were observed.

Study by [42], observed increases in the frequency of chromosome aberrations in the peripheral lymphocytes after repeated contraceptive pills (26).

The results of total comet score in the study of [44] revealed high significant difference between the studied hairdressers and their controls in DNA damage (Comet score  $161.15 \pm 45.84$ ) compared to ( $93.6 \pm 20.13$ ) respectively. These results matched that of

Galiotte et al., 2008 [45], where there was a significant difference between Comet score of hairdressers and their controls with mean total Comet score  $159.8 \pm 71.0$  and  $125.4 \pm 64.1$ ; respectively. Also, Cho et al., 2003 [46], proved that acute exposure to contraceptive pills causes DNA damage in human lymphocytes by means of the Comet assay. [47], reported that there is a statistically significant dose dependent association between contraceptive pills and risk of ovarian cancer. Moreover, [48], proved that adherence to safety precautions for the handling of contraceptive pills offers sufficient protection against local and systemic exposure and thus against their occupational health hazards. Overall, the data indicate higher risk of cancer after exposure to contraceptive pills depending on concentration and exposure time

#### Histopathological study.

Current results, demonstrates a histological sections for (heart,liver, ovary, intestine, lung, and kidney) in rabbits groups treated with contraceptive pills at (1000 $\mu$ g/ml per day) using (H&E, X200).Figure(1) showed pathologic changes in the intestine of treated rabbits include epithelial erosion, PMN infiltration referring to acute inflammation, as well as, distortion shape. This phenomena which called Pautz-syndrome, its accrue in the intestine when the body exposed to carcinogenic material, when orally intake degraded by juice and loss its advantage where converted to carcinogenic material, (42) referred to that the Macrovesicular fatty change associated with metabolic disturbances and is

generally readily reversible, whereas microvesicular fatty change is more likely a reflection of toxicity Only a minimum portion of the absorbed contraceptive pills was traced in serosal fluid at the end of 3 h incubation period, while most of it was still present in intestinal tissue. The relatively lesser recovery of contraceptive pills in its native form suggested transformation of this compound in the intestine to a certain extent during its absorption.

Histopathological analysis was carried out by hematoxylin/eosin method of the paraffin jelly rolled liver. Samples presented with high-grade dysplasia (pre-cancerous lesion), highly infiltration of RBCs (blood lysis), also section exhibit fatty degeneration (figure2), this changes due to same reasons for Pautz-syndrome (40), where exposure to carcinogenic material causing genetic defect in chromosome19, the gene known as STK11 (LKB1)(43) is a possible tumor suppressor gene (44).The distribution of contraceptive pills in the various tissues namely, lung and heart is shown in figure (3). Upon oral administration of contraceptive pills to rabbits group showed blood lysis, distortion of shape, air vacuoles formation, in lung tissues. Recently, heart section revealed highly blood lysis(figure4). These results came in harmony with(45), they showed peak concentration of contraceptive pills was observed at 6 h, after oral administration at dose (1000 mg/kg).

The results, demonstrates a histological sections for (ovary) in rabbits groups treated with contraceptive pills at (1000 $\mu$ g/ml per day) using (H&E, X200).Figure(5) showed pathologic changes include fibrosis, extensive

collagen deposition, blood lysis, where there's highly RBCs infiltration in tissue, as well as, follicular formation. While, figure (6) showed histopathological change in kidney includes blood lysis, and highly infiltration of inflammatory cells in tissue. Research studies causing great concern include those that have indicated an increased incidence of venous thrombosis due to increased blood clotting related to the use of OCs. Both high and low doses of OCs have been linked with a risk of thromboembolic disease ("Should oral contraceptives be sold over the counter", 2008). Hormonal treatments, such as oral contraceptives have been linked to an increased risk of atrial complications as well (19, 2007). This increase in blood clotting is said to cause other problems, including myocardial infarction, stroke, and hypertension (Akhigbe et al, 2008). The increased risk of these events seems to be caused by an increase in the procoagulant factors needed for blood clotting, including fibrinogen and factors VII, IX, X, and XII.

According to 19, the use of OCs has caused an increased occurrence of venous thromboembolism during the first years of use, specifically targeting susceptible groups (19). goes on to say that an increase in the dosage of oral estrogens clearly affects hemostasis and causes a significant increase in the risk of venous thromboembolism. The dose of estrogen in oral contraceptives is much higher than in hormonal treatment for menopausal women, thus increasing the clot risk for women on OCs (19). Even at low dosages, these oral estrogens found in many OCs reduce blood levels of coagulation

inhibitors, increasing the rate and speed of coagulation, thus causing an increased risk of venous thromboembolism. This unfortunately shows that even decreasing the amount of estrogen in OCs will not have much effect on improving the risk ratio (19). While combining progestins with estrogen can counteract some of the effects of estrogen only use, the risks for venous thromboembolism still remain. In fact, some studies have shown that recent generations of combined OCs may have a higher risk of venous thrombosis than earlier generation combinations (44).

Another study by Mirjam Meltzer and colleagues shows the correlation in women with hypofibrinolysis (decreased ability to dissolve a blot clot) and the increased risk of a venous thrombosis imposed by the use of OCs. In this study, women with hypofibrinolysis who also used oral contraceptives had a 22% increased risk of developing a venous thrombosis, compared to the women who were not using OCs (22).

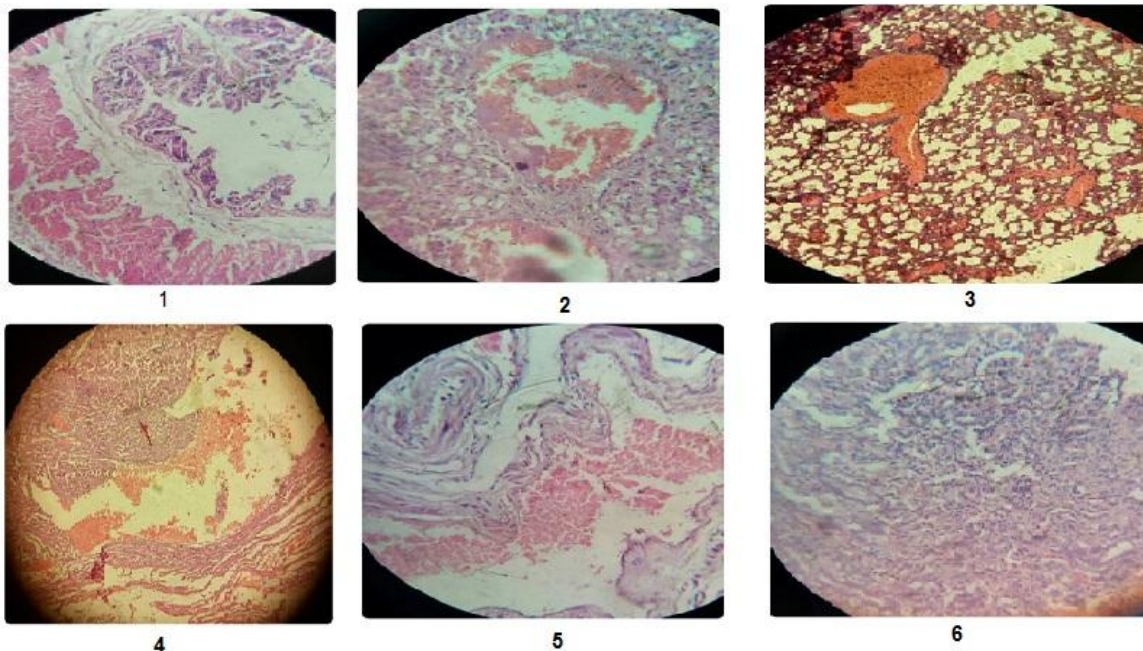
While many studies have published the benefits that the use of oral contraceptives can have on decreasing the risk of many types of cancers, research has shown that the use of the pill can also increase the risks of developing certain other types of cancer.

This study found that women who used oral contraceptives for more than eight years had an increased risk of being diagnosed with cancer, specifically cervical cancer and cancer of the central nervous system or pituitary ("Use of the pill can offer cancer protection", 2007) ("Do women who have ever used OCs have an

increased risk of cancer", 2008). A study by the Oxford Family Planning Association correspondingly discovered an increase in mortality rates among women using oral contraceptives for greater than eight years compared to those who were considered to be never-users (24). There has also been a correlation between the use of oral contraceptives and an increase in thyroid cancer risk ("Correlation with Cancer Risk", 2008). This study on women with thyroid cancer showed that risk for thyroid cancer greatly increased with the use of progesterone found in OCs.

The risk of thromboemboli caused by the use of OCs causes an increase in other cardiovascular events such as strokes and myocardial infarctions. For this reason, women at risk or with a history of coronary artery disease, hypertension, and current smokers should not take oral contraceptives. It is the increased androgen activity that has been

associated with increased hypertension, diabetes, and cardiovascular mortality. According to one study, users of second-generation oral contraceptive had an increased risk of myocardial infarction (19). Women with a history of migraines have an increased risk of ischemic stroke, with the odds ratio increasing from 6.2 to 13.9 (25). According to Dr. Alfonse Masi, numerous clinical reports suggest that using oral contraceptives may result in neurologic complications- including those of the retinal arteries, vascular headaches, and other cerebrovascular disorders (46). Studies on the third-generation contraceptives are still inconclusive as to whether or not they have an increased risk of causing strokes. However, all studies, regardless of the generation of oral contraceptive used, have concluded that women who smoke and use oral contraceptives have a greater risk of developing cardiovascular problems.





## Conclusions

The present study revealed Contraceptive pills at concentration (1000µg/ml) had highly apoptotic effect on the human proliferative lymphocytes. As well as, it increase their size and change their morphological shape, also, induced highly DNA fragmentation in blood lymphocyte depending on the dose and exposure time. On the other hand, it's a highly mutagenic material, might be developed carcinogenic effects associated with long term of using that caused increasing in proliferation cells furthermore it seemed had highly side effect more than its beneficial role. Contraceptive pills revealed highly degree of histopathological effect on all internal tested organs (ovary, liver, heart, kidney, and intestine).

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