Investigation of *In-ovo* cytogenetic effect of Levofloxacin

Marwa, H. N. and Falah, M. K. AL-Rekabi

Department of Physiological and Pharmacological, Collage of Veterinary Medicine, Baghdad University, Iraq.

E-mail: mr-hamoody77@yahoo.com

Summary

This study was carried out to investigate the cytogenetic effect of Levofloxacin after *In-ovo* inoculation. Forty eight fertilized eggs were used, divided equally into eight groups and inoculated through shell puncture above air cell with materials in equestion and incubated for two different periods three and seven days. They were assigned as group one and group two 10 µg Levofloxacin, group three and group four 20µg levofloxacin, group five and group six considered positive control 0.05 µg Mitomycin-C, group seven and group eight (Phosphate Buffer Saline) considered negative control groups. All eggs from *in-ovo* experiments were subjected to cytogenetic tests, such as Mitotic Index, Replicative Index and Sister Chromatid exchange. Eighty fertilized eggs were handled with the same manner of *in-ovo* inoculation and used for calculation Hatchability index and weekly body weight. The results revealed significant (P<0.05) increases in Mitotic Index and Replicative Index of all groups treated with levofloxacin compared to positive control, but there were significant (P<0.05) decreases compared to control negative. There were no significant changes in Sister Chromatid exchange of all treated levofloxacin *in–ovo*. The hatchability index revealed significant (P<0.05) decreases in group three which was inoculated 20 µg levofloxacin after three days of incubation compared to all Levofloxacin treated groups and both positive and negative control groups. In conclusion, levofloxacin *in–ovo* inoculation has no substantial cytogenetic effects.

Keywords: In-ovo, Cytogenetic, Levofloxacin, Mitotic index.

Introduction

Levofloxacin which has trade names such as levaquin (US), tavanic (EU), is a broad spectrum antibiotic of the fluoroquinolone medicine class (1). Levofloxacin is frequently prescribed empirically for a wide range of infections such as pneumonia and urinary tract infection before the causal organism is known (2). Compared to earlier antibiotics of the fluoroquinoline class such as ciprofloxacin, levofloxacin, it exhibits greater activity towards gram-positive bacteria, but lesser activity against gram-negative especially *P. aeruginosa* (3). Levofloxacin is associated with an elevated risk of musculoskeletal injury in children (4). Since levofloxacin is widely used in poultry industry in the following bacterial infections sinusitis, bronchitis, community acquired pneumonia, yellow and white dysentery, enteritis, pericarditis, Ovarian inflammation, green suppuration bacillus, Staphylococcus infection, cholera, Escherichia coli, green suppuration bacillus, skin infections, urinary tract infections and acute pyelonephritis **(5 and 6)** presence of residuals in their various of poultry tissue like meat, liver, kidney, eggs and products, will cause several alteration in health effect of consumers such as gastrointestinal disturbances such as nausea, vomiting and diarrhea (7). At slightly higher doses, CNS signs of dizziness, restlessness, headache, depression, somnolence or insomnia may be seen (8). However the safety of repeated oral administration of levofloxacin in the poultry is not well documented because of limited data (9). People are consuming chicken and eggs as daily food, so they get small doses of the drugs that may cause some side effects. The residual of levofloxacin in poultry products such as egg may be consume by human and exert some deleterious effects. Thus is experiment was designed to evaluate the probable cytogenetic and mutagenic effect of levofloxacin in eggs of layers as a warning for public health and poultry industry due to its   wide range uses.

Materials and Methods

Eggs were purchased from Hendrix Genetics Company, France, cleaned and free

of any abnormal external appearance like fissures or cracks or rough shell and subjected immediately for In-*ovo* cytogenetic study. Levofloxacin 5% was obtained from Travipharma company-Holland, Bromodeoxy uridine (Brdurd) obtained from Serva company-Germany, Colchicine from Houde company-French; Glacial acetic acid (99.99%) from BDH company-England, Methanol absolute from Hayman company-England, Mitomycin–C (MMC) from MHECO company-China, Phosphate Buffer Saline (PBS) from BDH company-England and Potassium chloride (KCL) from BDH company-England.

In 1st experiment forty eight embryonated eggs were divided equally into eight groups and inoculated with materials in question through air cell after 3 and 7 days of incubation of eggs under optimum condition. They were assigned into eight groups G1, G2, G3, G4, G5, G6, G7 and G8 inoculated 10µg Levofloxacin (G1 and G2), 20µg Levofloxacin (G3 and G4), 0.05 µg Mitomycin-C (G5 and G6) considered positive control groups while (G7 and G8) inoculated 0.1 ml Phosphate buffer saline (PBS) and considered negative control groups (Mitomycin-C inhibits DNA synthesis it reacts covalently with DNA, in vivo and in vitro, forming cross links between the complementary strands of DNA. This interaction prevents the separation of the complementary DNA strands, thus inhibiting DNA replication), Mitomycin-C 0.05 µg and 0.1 ml Phosphate buffer saline receptively. Harvesting the chick embryos according to the procedure of (10) after 72 hr. of incubation of fertilized eggs, the air sac of each egg was determined by candle, and punctured by needle in order to inoculate with subjected materials to be absorbed by the embryo through the inner membrane of the egg. Each egg was inoculated with Brdud 150 µg/ egg and optimized the size of 0.1 ml; then the hole was closed by wax and re- incubated. After two hours (74 hr. of incubation) of the Bromodeoxy uridine (Brdurd) (detection of proliferating cells in living tissues) inoculation, the wax was removed and the eggs inoculated with Levofloxacin that is required to test with optimize volume 0.1 ml and the hole was re-covered again with wax,

and re-incubated. After 24 hr. of Levofloxacin inoculation (98 hr of incubation), the wax was removed and the eggs inoculated with 10 µg/egg Colchicine (to stop mitosis) with optimize volume 0.1 ml, the hole was re-covered with wax again and re-incubated. After 2.5 hr. of incubation, the eggs were broken down and the embryos obtained and were put in test tube containing 5 ml of warm solution KCL (0.75 M). The embryos were macerated fairly by Pasteur pipette and incubated in water bath with 37°C for 45 min. with continuous stirring each 5 min. The cells of embryos were obtained through centrifugation of tubes with speed 2000 rpm/ 10 min.

The supernatant was neglected and the precipitated (cells of embryos) was taken. The fixative (3:1 methanol - acetic acid volumes) was added gradually drop by drop with continuous mixing till 5 ml volume in order not to get a conglomerate of cells and preserved embryo cells. Slides which were selected for mitotic index calculation and Chromosomal aberrations were stained with freshly prepared Giemsa stain for 15 min., and washed with distilled water and left to dry at room temperature. Microscope examination was done under 40x and 100**x** objective lens. Mitotic index was estimated (MI **%**) by calculation the metaphase in 1000 divided cells according to the following equation (11).

$$\text{MI\%} = \frac{\text{Number of divided cells}}{1000 \text{ cell}} \times 100$$

To calculate the Replicative index (RI) and Sister chromatid exchange (SCE), samples were stained by Hoechest dye 33258 and Giemsa stain according to Goto (12). The slides examined by a microscope of strong magnification 100x (oil emersions). Replicative index (RI **%**) was calculated in 100 cells in metaphase for three cell cycles according to the following equation (12). Sister chromatid exchange was also calculated in each 50 cells (in metaphase).

$$\text{RI} = \frac{\text{M1} \times 1 + \text{M2} \times 2 + \text{M3} \times 3}{1000} \times 100$$

Examination the slide was done under florescent microscope staining in Acridine orange stain solution (0.01 %) for 4 to 5 min. Rinse slides in Sorensens buffer (0.06M, pH

6.5) for 1 min. Mount the slide in Sorensen's buffer (0.06M, pH 6.5) and examined using a wave length of 450 to 500 nm.; SCE was calculated in 50 cells passed in the second division, but only in first seven pairs of chromosomes (13). Same producers which was used in obtaining embryonic tissue after 3 days of incubation except that the inoculation of Brdurd and was inoculated at the day sixth of embryonic life (after 144 hrs. of inoculation).

In 2nd experiment eighty embryonated eggs divided equally into eight groups and assigned to G1, G2, G3, G4, G5, G6, G7 and G8 they were subjected to same manner of treatment in 1st experiment. The eggs incubated under the optimum condition to full term incubation (21 days). Hatchability index was calculated by divided hatched eggs by the total number of each group multiplying with 100.

All data were expressed as Mean ± Stander error and analyzed by one way analysis (ANOVA) test (SPSS). A p value <0.05 was considered statically significant. LSD multiple range test were used for comparing between means (14).

Results and Discussion

The results showed significant increases (P<0.05) in Mitotic index of all treated groups with Levofloxacin, G1, G2, G3 and G4 compared to mitotic index of G5 and G6, but this increase is non-significant compared to mitotic index of G7 and G8 (negative control PBS 0.1 ml -3 days), (Table, 1) and (Fig. 1, A and B) which represent mitosis).

Table, 1: Mitotic index of chick's embryo in two different stages (3 and 7 days of incubation) exposed to two different concentrations of Levofloxacin.

Groups	Mean ± SE
G1:Levofloxacin 10µg -3days	3.075 ± 0.788 /AB
G2:Levofloxacin 10µg -7days	3.100 ± 1.002 AB
G3:Levofloxacin 20µg -3days	3.925 ± 1.034 AB
G4:Levofloxacin 20µg -7days	5.250 ± 1.400 A
G5:Mitomycin -C 3days (control positive)	1.775 ± 0.275 BC
G6 Mitomycin -C 7days (control positive)	1.525 ± 0.252 C
G7:Phosphate buffer solution 3days (control negative)	5.200 ± 1.364 A
G8: Phosphate buffer solution 7days (control negative)	4.425 ± 1.172 A

* LSD=2.4, *Different capital letters denote significant (P<0.05) differences among groups.
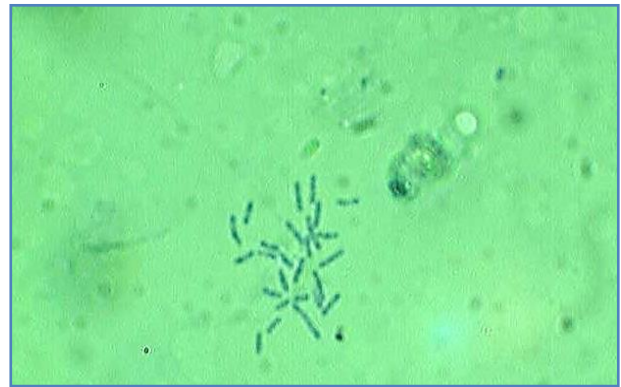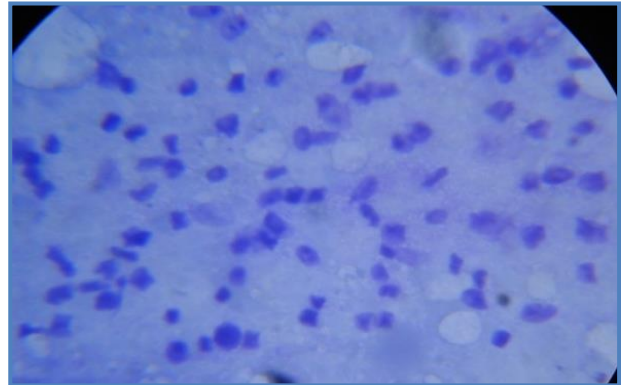
Figure, 1: A. Mitosis of chick embryo cells (Acridine stain under fluorescent microscope PH 1.9 X)   B. Mitosis of chick embryo cells (Hoechest stain 100x under light microscope).

There was a significant increase (P<0.05) in replicative index of all Levofloxacin treated groups, G1, G2, G3 and G4 compared to replicative index of both positive control G5 and G6 and negative control G7 and G8. The increase in replicative index was more prominent in groups treated with levofloxacin 20 µg/egg for two different stages of incubation 3 and 7 days respectively, (Table, 2) and (Fig. 2) are clarified different stages of cell cycle mitosis (M1, M2 and M3).
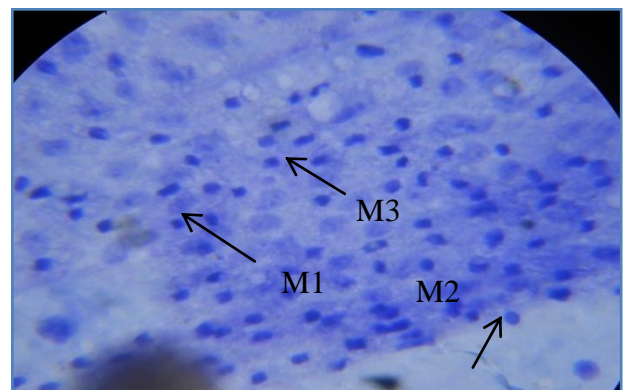
Figure, 2: Chick embryo cells treated In-ovo with 20µg levofloxacin after 3 days of incubation (observe M1, M2 and M3 stages of mitosis) (Hoechest stain 100x under light microscope).

Table, 2: Replicative index of chick's embryo in two different stages (3 and 7 days of incubation) exposed to two different concentrations of levofloxacin.

Groups	M1 Mean	M2 Mean	M3 Mean	Replicative Index %
G1- 3days Levofloxacin 10µg	2.34	1.56	1.50	0.996 DE
G2- 7days Levofloxacin 10µg	2.38	6.67	0.91	1.840 B
G3- 3days Levofloxacin 20µg	8.38	5.61	1.75	2.485 A
G4-7days Levofloxacin 20µg	8.61	4.04	1.95	2.596 A
G-3days mitomycin (control positive)	1.75	1.30	1.00	0.735 GF
G6-7days mitomcyin (control positive)	2.74	1.56	0.60	0.766 F
G7-3days Phosphate (control negative)	7.25	2.65	1.42	1.681 BC
Group8-7days Phosphate (control negative)	6.31	2.55	1.35	1.048D

*LSD=0.12, Different capital letters denote significant (P<0.05) differences among groups.

The SCE which was observed in all levofloxacin treated groups (G1, G2, G3 and G4) had not statically significant P<0.05 differences compared to SCE of both control negative groups (G7 and G8) which was inoculated with PBS, but there were significant P<0.05 decreases compared to both positive groups (G5 and G6) which was inoculated by Mitomycin-C. (Table, 3) and (Fig. 3 and 4) represented SCE.

Table, 3: Sister chromatid exchange of chick embryo cells in two different stages of incubation (3 and 7 days) exposed to two different concentrations of levofloxacin.

Groups	SEC Mean ± SE
G1:Levofloxacin 10µg -3days	1.100 ± 0.707 B
G2:Levofloxacin 10µg -7days	1.550 ± 0.478 B
G3:Levofloxacin 20µg -3days	1.200 ± 0.577 B
G4:Levofloxacin 20µg -7days	1.250 ± 0.946 B
G5:Mitomycin -C 3days (control positive)	3.660 ± 0.478 A
G6 Mitomycin -C 7days (control positive)	3.500 ± 0.500 A
G7:Phosphate buffer solution 3days (control negative)	1.750 ± 0.629 B
G8: Phosphate buffer solution 7days (control negative)	1.660 ± 0.816 B

*   LSD=1.7
** Different capital letters denote significant (P<0.05) differences among groups.
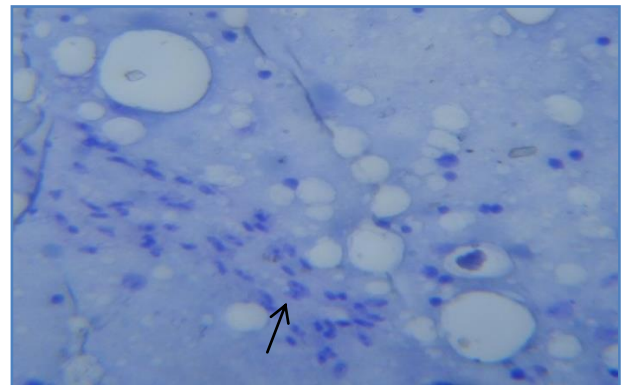
Figure, 3: Sister chromatid exchange in chick embryo cells after 20 µg Levofloxacin in-ovo inoculation after 7 days of incubation (Hoechest stain 100x under light microscope)
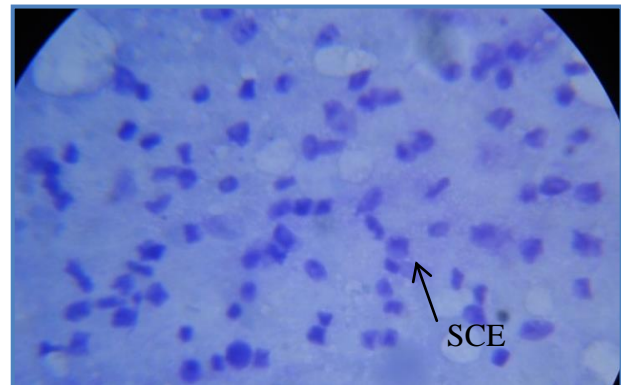
Figure, 4: Sister chromatid exchange in chick embryo cells after 10 µg levofloxacin in-ovo inoculation after 7 days of incubation (Hoechest stain 100x under light microscope)

There were significant decreases (P<0.05) in hatchability index of G1, G3 and positive control G5 compared to the G7 and G8. Both G3 and G5 also showed significant decrease (P<0.05) in hatchability index compared to G2, G4, G6 and G8, while G1 showed no significant differences (P<0.05) in hatchability index compared to G2, G4, G6 and G8 (Table, 4).

Table, 4: Hatchability index of chick embryos exposed to two different concentrations of levofloxacin in two different stages (3 and 7 days) of incubation.

Groups	Hatchability index%
G1:Levofloxacin 10µg -3days	60 BD
G2:Levofloxacin 10µg -7days	70 AB
G3:Levofloxacin 20µg -3days	50 D
G4:Levofloxacin 20µg -7days	70 AB
G5:Mitomycin-C 3days (control positve)	50 D
G6: Mitomycin-C 7days (control positive)	70 AB
G7:Phosphate buffer solution -3days (control negative)	80 A
G8: Phosphate buffer solution 7days (control negative)	70 AB

*LSD=15.5
**Different capital letters denote significant (P<0.05) differences among groups.

The MI is a cytogenetic test that is used both in vivo and in vitro to characterize proliferating cells and identify compounds that inhibit or induce mitotic progression (15). Therefore, by the employment of this assay the effect of different physical and chemical agents on the mitotic response can be detected, whether there are negatively or positively by chemicals, radiations, drugs and medicinal plants (16). The mitotic index assay is used to characterize proliferating cells and to identify the compounds that inhibit mitotic progression resulting in a decrease in the MI of that population. Mitotic abnormalities often arise directly from defects of centrosome and /or mitotic spindles, which then induce prolonged mitotic arrest or delayed mitotic exit and trigger induction of apoptosis (17).

Depression of the mitotic index is usually a consequence of a reduced rate of cell proliferation (18). The finding of present study shows that the Levofloxacin used in *Ovo*-inoculation showed significant increases in MI of all treated groups compared to positive control group Mitomycin-C (7 days), but there were no significant increases in mitotic index compared to positive control group Mitomycin-C (3 days) and negative control groups of PBS (3 and 7 days). Levofloxacin caused elevation in mitotic index that indicated more cells divide and mean more cells division. The increasing of mitotic index obvious in cancer cells may be elevated during necessary processes to life, such as the normal growth of plants or animals, as well as cellular repair the site of an injury (19). Compared to danofloxacin of same generation, which causes decrease in mitotic index at 7 days of age of chick embryo, levofloxacin showed no changed in index in different concentration and periods of incubation, but showed slights increase after 7 days in *ovo*-inoculation; this is related to development of liver and it is differentiated in chick embryo at 7 days of age and increase in metabolic activity and detoxification (20).

Compared to Mitomycin-C that showed decreases in mitotic index because of absence of metabolic activation (21), and this is agreement with (20) who showed that all substances that act directly, indirectly and their metabolic products are effective in the embryo at 7 days of age more than in age of 3 days due to the developed of liver at day 7 of age. The RI is an index of cytokinetics in cultured cells, determining the RI in human blood cultures may be useful in gene toxicity testing, because inhibition of DNA synthesis will result in a decrease of the RI (22). The finding of present study indicates that levofloxacin in *Ovo*-study showed significant increases in the replicative index of all treated groups compared to both negative control groups (PBS) and positive control groups and these increases are more prominent in replicative index of group treated with Levofloxacin 20 µg 3 and 7 days. The levofloxacin caused an increment cells division and this is increased which is necessary for normal growth as well cell repair (19).

These changes also could be attributed to the liver in chick embryos at seven day of age and ability to detoxification and metabolic activity (20). The fluoroquinolones are the only direct inhibitors of DNA synthesis by binding to the enzyme-DNA complex, they stabilize DNA strand breaks created by DNA gyrase and topoisomerase IV. Ternary complexes of drug, enzyme, and DNA block progress of the replication fork. Cytotoxicity of fluoroquinolones is likely a 2-step process involving (1) conversion of the topoisomerase-quinolone-DNA complex to an irreversible form and (2) generation of a double-strand break by denaturation of the topoisomerase (23). Compared to danofloxacin and enrofloxacin where they decrease in RI of chicks embryos in 3 and 7 days may be due to their inhibitory effect on the DNA synthesis lead to decreases in replicative of DNA, such as drugs hycanthon and praziquntal which were used in mice caused decrease in RI of bone marrow due to effect on DNA (24).

Also ciprofloxacin causes decreased replicative index, inhibit DNA gyrase and topoisomerase II, which is important for resolving the super helical intertwined structure of DNA during replication (25). It is also interfered in segregation of chromosome at anaphase in mitosis consequently resulting in the delay of cell cycle (26). Our study revealed that levofloxacin influence on cell cycle progression through their effect on the percentage of cells that pass through M1, M2

and M3 and replicative index of cell through inducing the DNA gyrase lead to more cells replicated and division (19). But our finding did not agreement these studies, particularly mitotic index decreasing while in our study increasing in mitotic index. The SCE test is a fine and relatively fast and easy detection method for DNA/ chromosome damage caused by carcinogenic and mutagenic substances (27). As a basic cytogenetic biomarker, the test is not only used for the assessment of the effect of genotoxic factors, but also for the prevention and monitoring of diseases (28 and 29).

The results revealed that Levofloxacin showed no significant differences in sister chromatid exchange of all in-*ovo* treated groups with the levofloxacin compared to negative control groups (PBS), but there was a significant decrease compared to sister chromatid exchange of both positive control groups inoculated with mitomycin-C 0.05 µg 3 and 7 days. SCE is usually a sensitive indicator of genotoxic effects than chromosomal aberrations (30). Our results revealed that levofloxacin caused no elevated in SCE and not cause damage to the strands of DNA and no chromosomal aberration. Since (30) were referred to that SCE generated by the abnormal recombination of double-strand DNA breaks occurring at the junctions between completely and partially duplicated replicon clusters. Thus, agents that induce absolute blocks to DNA fork displacement will favor the appearance of SCEs because double-strand breaks have more time to occur at junctions. Mitomycin–C which cause decrease in sister chromatid exchange by inhibitory effect on DNA, that prevents the separation of the two strains of DNA during the replication process (31). The inhibitory effect of mitomycin-C on Human lung adenocarcinoma (A549 cells) was obtained mainly by cell cycle retardation (cell growth extension and proliferation deceleration) rather than cell apoptosis, clear increase of G1/G0 cells and decrease of S and G2/M cells, the inhibitory effect of mitomycin-C might be mainly attributed to cell arrest in G1 phrase (32), mitomycin-C needs metabolic activation to exert it's effect (33).

Pathogenic agents can decrease the hatchability rate during embryonic development and embryonic death causes serious economic losses to the poultry industry (34). For many years, researchers have been using different antibacterial compounds to restrict pathogens and enhance the performance of different poultry species, including young chicken, quail, turkey, broiler and layers chickens (35). The significant decreases in hatchability index of chick embryos and this decrease in hatchability index, it may be related to many factors cause stress to embryo and then death, such as the methods of injection of antibiotics and open and close the eggs shell, this agreement with (36 and 37) they were referred to that some injection sites that are present in fertile eggs at day 4 of incubation are the air cell and yolk sac, injection antibiotics into the air cell of the egg is discontinued and is not suitable for breeding purposes because drastic mortality of embryos occur when eggs treat by this procedure, therefore decreases in hatchability index it might be not related to the injection of Levofloxacin in inside eggs only. Also injection high doses of antibiotics to egg were is toxic to the embryo such as tylosin when used in high doses (38).

According to (39) solutions at high concentrations may affect egg osmotic balance and consequently embryo development. This is in somehow matching our finding of decrease hatchability index especially in egg inoculated after 3 days of incubation, but not after 7 days of incubation. This might be due to parent Levofloxacin when the liver of embryo is not yet developed in this day of age. In Conclusions: Levofloxacin in-*ovo* inoculation has no substantial cytogenetic effects.

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دراسة التأثير الخلوي لعقار الليفوفلوكساسين داخل البيض

مروى حسين نصيف و فلاح موسى كاظم الركابي

فرع الفسلجة والأدوية، كلية الطب البيطري، جامعة بغداد، العراق.

E-mail: mr-hamoody77@yahoo.com

الخلاصة

صُمِّمت هذة الدراسة لتقييم التأثير الخلوي الجيني لعقار الليفوفلاكساسين أحد أفراد الفلوروكينولونات في البيض. استخدمت ٤٨ بيضة مخصبة وقسمت بالتساوي إلى ثماني مجاميع، وعُوملت الأجنة مباشرة حيث حقنت المواد بإحداث ثقب في قشرة البيضة فوق الفسحة الهوائية مباشرة، حيث حقنت المجموعتان الأولى والثانية ١٠ مايكروغرام من الليفوفلاكساسين والمجموعتان الثالثة والرابعة ٢٠ مايكروغرام من الليفوفلاكساسين والمجموعتان الخامسة والسادسة (السيطرة الموجبة) مايتومسين-C ٠،٠٥

مايكروغرام والمجموعتان السابعة والثامنة (السيطرة السالبة) اعطيت دارئ الفوسفيت وحضنت البيوض لمدتين مختلفتين (ثلاثة وسبعة أيام . حيث دُرست الاختبارات الخلوية في البيض وهي معامل الانقسام الخلوي، معامل التضاعف الخلوي واختبار التبادل الكروماتيدي الشقيقي.استخدمت ثمانون بيضة مخصبة وعُوملت بنفس الطريقة السابقة لحساب معدل الفقس والزيادة الوزنية بالاسابيع. اظهرت النتائج زيادة معنوية في معاملي الانقسام الخلوي والتضاعف الخلوي لكل المجاميع التي اعطيت اليفوفلوكساين مقارنة مع مجاميع السيطرة الموجبة،لكن هناك نقصانا على مستوى $P<0.05$ معنويا مع مجاميع السيطرة السالبة ولا يوجد أيّ تاثير معنوي في اختبار التبادل الكروماتيدي الشقيقي لكل المجاميع التي أعطيت اليفوفلاكساين مقارنة مع مجموعة السيطرة السالبة داخل البيض. أظهرت النتائج نقصاناً معنوياً في أوزان الأفراخ التي أعطيت اليفوفلاكساسين مقارنة مع مجموعة اليسطرة السالبة أما معدل نسبة الفقس فقط اظهرت النتائج نقصانا معنويا في المجموعة الثالثة التي أعطيت ٢٠ مايكروغرام من ليفوفلاكساسين وحضنت لمدة ثلاثة أيام مقارنة مع جميع المجاميع التي أعطيت ليفوفلاكساسين ومجموعة السيطرة السالبة والموجبة.

الكلمات المفتاحية: في البيضة، خلوي، ليفوفلاكساسين، معامل الإنقسام الخلوي.