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The Pesticides Cypermethrin and Signum: Potential Genotoxicity and Effects on Reproductive Biology of Female Albino Rats

المبيدان سابيرميثرين وسيغنوم: سُمّيتهما الجينية وتأثيرهما على بيولوجيا التناسل لإناث جرذان التجارب

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in "*Environmental Biology"* from the Faculty of Graduate Studies at Birzeit University, Palestine.

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DECLARATION

This is my original work and has never been submitted in part or whole for an award in any institution.

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Date: 4/5/2021

DEDICATION

This is dedicated to my family and friends, especially to my mother who is always there for me, for her encouragement and supportive words.

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LIST OF ABBREVIATIONS

AF: Atretic Follicle

ALP: Alkaline Phosphatase

ALT: Alanine Transaminase

C: Control

CAT: Catalase

CL: Corpus Luteum

CYP: Cypermethrin insecticide

CYP1: 10mg/kg bw Cypermethrin

CYP2: 20mg/kg bw Cypermethrin

DDT: Dichlorodiphenyltrichloroethane

EDCs: Endocrine Disruptors

FSH: Follicle Stimulating Hormone

GGT: Gamma Glutamyl Transferase

GST: Glutathione S-Transferase

LH: Luteinizing Hormone

MDA: Malonate dehydrogenase

MIX1: 10mg/kg bw Cypermethrin+10mg/kg bw Signum

MIX2: 20mg/kg bw Cypermethrin+20mg/kg bw Signum

MRLs: Maximum Residue Levels

PF: Primary Follicle

PND: Post Natal Day

Q_oI: Quinone Inhibitors

ROS: Reactive Oxygen Species

SDH: Succinate Dehydrogenase

SOD: Supper Oxide Dismutase

SF: Secondary Follicle

SIG: Signum fungicide (Boscalid+ Pyraclostrobin)

SIG1: 10mg/kg bw Signum

SIG2: 20mg/kg bw Signum

TF: Tertiary Follicles

The Pesticides Cypermethrin and Signum: Potential Genotoxicity and Effects on Reproductive Biology of Female Albino Rats

Abstract

Pesticides are considered among the most heavily worldwide used chemicals that pollute the environment. Their residues endanger human and animal health can be found in the environment, as well as in a variety of plant and animal products. Pesticides can be genotoxic and can affect hormonal balance in animals. To combat insects and fungal diseases in agriculture, most farmers spray a mixture of an insecticide along with a fungicide. Cypermethrin (an insecticide) and Signum (a fungicide) are globally among the most widely-used insecticides. In the West Bank and Gaza, significant amounts of pesticides, including Cypermethrin and Signum, are used, some of which are internationally banned.

The present study aims at evaluating the effects of different concentrations of the two pesticides, Cypermethrin and Signum, and their mixtures on the reproductive biology of female albino rats and evaluating their potential genotoxicity using Randomly Amplified Polymorphic DNA (RAPD) Technology. Female albino rats were subdivided into 7 groups (control "C", 10mg/kg.bw Cypermethrin "CYP1", 20 mg/kg.bw Cypermethrin "CYP2", 10 mg/kg.bw Signum "SIG1", 20 mg/kg.bw Signum "SIG2". In addition, a mixture of 10 mg/kg.bw Cypermethrin and 10 mg/kg.bw Signum "MIX1" and another mixture of 20 mg/kg.bw Cypermethrin and 20 mg/kg.bw Signum "MIX2") were used. Each female in the 7 groups was given 0.5 ml of the appropriate treatment by gavage 5 days a week. Female rats were subjected to treatments from day one of the experiment to day 21 after birth (about 42 days). After birth, female pups (F1), whose mothers continued to receive the appropriate dose, were also studied.

Results indicated that the pesticides and their mixtures did not show any significant impact on the percentage of conceiving females in different groups. The percentage of conceiving females was ranging between 75-100%. In addition, during the experiment, there was no significant difference between the weights of female rats receiving different treatments compared to the control group. Moreover, at birth, mean weights of pups in each group did not show any significant difference. However, at the age of 21 days, only weights of MIX1 pups showed significant decrease in weight

compared to the control. The mean number of offspring/female in each group showed no significant difference and ranged between 6-8.8 pups. These results indicate that, under the conditions of the present experiment, there was no, or little impact, of the two pesticides and their combinations on conceiving, weight, number of offspring and weight of offspring.

Hormonal analysis of female albino rats at the end of the exposure period (day 42), showed that progesterone and FSH levels in the control and treatment groups did not show any significant difference. This indicates no impact of CYP and SIG and their combinations on these two hormones. Levels of LH in the control female groups were significantly higher than those of all groups, except SIG2. This indicates that the CYP and SIG pesticides and their combinations interfere (by reduction) with LH levels. Estradiol levels in CYP2 group were significantly higher than those of the Control and some other groups. This also might indicate an alteration of estradiol levels by CYP but not SIG.

Hormone analysis of female offspring (pups) showed that levels of LH did not show any significant difference between all treatments and the control. Progesterone levels in CYP2 group was significantly higher than those of the control group. CYP1 group showed significantly less levels of estradiol than the Control group. FSH levels in all groups (except CYP1) were significantly less than those of the Control group. These results show interference of CYP with levels of progesterone, estradiol and FSH in female pups whose mothers received pesticides during pregnancy and lactation. Besides, results of hormonal analysis in both female mothers and their offspring indicate that CYP is a stronger hormonal disruptor than SIG.

Histopathological examination of the mothers' ovarian tissues exposed to CYP2 and SIG2 appear to have less number of normal follicles (9.3 and 12, respectively) and more unhealthy-looking tissues than the control group (25.7 normal follicles). Besides, in MIX 2 group the ovary size was very small, almost half the size of the control and other groups, with a reduced number of normal follicles (13.5). In addition, the control group had significantly higher mean number of total follicles compared to the treatments. Histopathological results of ovarian tissues in females subjected to CYP2 indicated less secondary and tertiary follicles compared to the control group, but similar number of primary follicle and corpus luteum. Besides, ovarian sections of the same group indicated the presence of congested blood vessels. Moreover, ovaries of mothers from groups exposed to CYP2, SIG2 and MIX2 show the development of micronuclei and vacuolated cells, while those of SIG2 group indicated a significant increase of atretic follicles (51%) compared to the control (15.5%). In general, both pesticides and their combinations were found to cause

many obvious histopathological disorders in mother females' ovarian tissues exposed to the higher dose of 20 mg/kg.bw.

In pups` ovarian tissues, there was no significant difference in the number of primary or secondary follicles between the four groups. However, the number of Graafian follicles was significantly reduced in CYP2 group (1.0) compared to the control (3.5). Besides, the control group had significantly higher mean number of total follicles compared to the treatments. In addition, the number of corpus lutei in SIG2 (0.5) was statistically less than that of the control (4.8). The female pups' groups showed an insignificant increase in the percentage of atretic follicles compared to the control. Generally, both pesticides at the higher dose of 20 mg/kg.bw caused some histopathological disorders in pups females that are related to total number of normal follicles, Graafian follicles and corpus lutei.

RAPD profiles generated from DNA obtained before exposure and after exposure revealed the formation of a total of 73 polymorphic bands representing around 25% of the total bands obtained after exposure. All groups, except the control, generated polymorphic bands that ranged between 6 (CYP1) and 22 (SIG1). The genetic similarity indices calculated for DNA profiles before and after exposure ranged between 94.28% (CYP 1) to 77.92% (SIG1). MIX1 showed a similarity index of 84.99% while MIX2 showed a similarity index of 82.15%. The average similarity indices of both doses together were 90.31, 84.91 and 83.57 for CYP, SIG and MIX, respectively. These findings indicate that both pesticides are genotoxic in all their treatments and doses. Besides, mixing of both insecticide and fungicides together increases their genotoxicity.

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ملخص

تعتبر مبيدات الآفات من أكثر المواد الكيميائية استخداماً على نطاق العالم والتي تلوث البيئة. يمكن العثور على بقايا من هذه المواد في المنتجات النباتية والحيوانية. تعد بقايا هذه المواد ضارة بصحة الإنسان والحيوان على حد سواء، حيث يمكن لها أن تؤثر على توازن الهرمونات في الحيوانات، وقد تؤدي إلى السمية الجينية. ولمكافحة الحشرات والأمراض الفطرية في الزراعة، يقوم معظم المزارعون برش خليط من مبيد حشري إلى جانب مبيدات فطري. يعتبر المبيد الحشري سايبرميثرين والمبيد الفطري سيجنوم من بين المبيدات الأكثر استخداماً على نطاق واسع على مستوى العالم. أما في الضفة الغربية وقطاع غزة، حيث تستخدم كميات كبيرة من مبيدات الآفات التي قد تكون محظورة دوليا، يعد السيبرميثرين والسيغنوم من المبيدات شائعة الاستخدام.

تهدف هذه الدراسة إلى تقييم تأثير تراكيز مختلفة من المبيدان سيبرميثرين وسيجنوم، كل على حدا أو مزيج منهما، على بيولوجيا التناسل لإناث فئران التجارب، بالإضافة إلى تقييم السمية الجينية للمبيدان باستخدام تقنية ال RAPD. لتنفيذ التجربة، تم تقسيم إناث الفئران إلى 7 مجموعات (المجموعة المرجعية "C"، مجموعة 10ملغ/كغم سيبرمثرين "CYP1"، مجموعة 20ملغ/كغم سيبرميثرين "CYP2"، مجموعة 10ملغ/كغم سيجنوم "SIG1"، مجموعة 20ملغ/كغم سيجنوم "SIG2"، بالإضافة إلى مجموعة مزيج من 10ملغ/كغم سيبرمثرين و 10ملغ/كغم سيجنوم "MIX1" ومجموعة مزيج من 20ملغ/كغم سيبرميثرين و 20ملغ/كغم سيجنوم "MIX2". تم اعطاء إناث الفئران في المجموعات ال7 الجرعة المحددة بالفم 5 مرات في الأسبوع بمقدار 0.5 مل في المرة الواحدة. استمرت إناث الفئران في اخذ الجرعات المحددة لمدة 42 يوم تقريبا، أي طوال فترة الحمل والرضاعة. تشير النتائج إلى أن المبيدان و مخاليطهما لا تظهران أي تأثير حقيقي على نسبة الحمل لدى إناث الفئران، حيث تراوحت النسبة من 75-100%. كذلك، لم يكن هناك اختلاف يذكر بين أوزان إناث الفئران اللاتي تلقين الجرعات المختلفة من المبيدات بالمقارنة مع المجموعة المرجعية. علاوة على ذلك، لم يظهر اي فرق يذكر في معدل أوزان الفئران حديثة الولادة. ولكن، ظهر هناك انخفاض في أوزان الفئران الصغيرة في المجموعة MIX1 في عمر ال21 يوم. كذلك، أظهرت النتائج عدم وجود اختلاف في متوسط عدد المواليد بين المجموعات حيث تراوح معدل عدد المواليد من 6-8.8 لكل أنثى. وبالتالي، وضمن ظروف التجربة

الحالي، تشير هذه النتائج إلى عدم وجود تأثير يذكر للمبيدين، منفردين أو ممزوجين، على حمل ووزن الفئران الكبيرة و ووزن وعدد المواليد.

أظهر التحليل الهرموني لأناث الفئران في اليوم 42 من التجربة أنه لم يكن هناك اي فرق بين مستويات البروجيستيرون و ال FSH في جميع المجموعات بالمقارنة بالمجموعة المرجعية، هذا يدل على عدم تأثير سيبرميثرين وسيجنوم ومخاليطهما على هذين الهرمونين. أما بالنسبة ل هرمون LH فقد كان مستواه في المجموعة المرجعية أعلى من مستواه في باقي المجموعات باستثناء SIG2. هذا يشير إلى أن سيبرميثرين ، سيجنوم و مخاليطهما تسبب انخفاضا في مستويات هرمون ال LH في الدم. كما أظهرت النتائج، أن مستوى الاستراديول في مجموعة CYP2 كان أعلى من مستواه في المجموعة المرجعية وبعض المجموعات الأخرى، مما يشير الى تأثير محتمل للسيبرميثرين على مستوى الاستراديول في الدم.

أظهر تحليل الهرمونات لإناث الفئران الصغيرة (الجراء) عدم وجود إختلاف واضح في مستويات LH بين المجموعات والمجموعة المرجعية، بينما أظهر مستوى مستوى البروجيستيرون إرتفاعا في مجموعة CYP2 مقارنة بالمجموعة المرجعية. أما هرمون الإستراديول فقد كانت مستوياته في المجموعة CYP1 أقل من المجموعة المرجعية. مستويات هرمون ال FSH أظهرت إنخفاضا في جميع المجموعات، باستثناء المجموعة CYP1، بالمقارنة مع المجموعة المرجعية. وتظهر هذه النتائج تأثير CYP على مستويات البروجستيرون والإستراديول في الجراء التي تلقت أمهاتها المبيدان أثناء الحمل والرضاعة .وبالإضافة إلى ذلك، تشير نتائج التحليل الهرموني في كل من الأمهات الإناث وذريتهن إلى أن CYP هو معطل هرموني أقوى منSIG .

أظهرت فحوصات الأنسجة لمبايض الأمهات المعرضة ل CYP2 و SIG2 انخفاضا عاما في عدد الحويصلات الطبيعية (9.3 و 12 على التوالي) مقارنة بالمجموعة المرجعية (25.7 حويصلة طبيعية)، هذا بالاضافة لوجود أنسجة غير طبيعية المظهر. أما المبايض في مجموعة MIX2، فقد كانت صغيرة الحجم بشكل لافت مقارنة بباقي المجموعات، حيث كانت تقريبا نصف حجم المبايض في المجموعة المرجعية. هذا بالإضافة إلى انخفاض عام في عدد الحويصلات الطبيعية (13.5). وبشكل عام، فقد كان معدل عدد الحويصلات الطبيعية في مبايض المجموعة المرجعية أعلى منها في باقي المجموعات. كذلك، فقد أظهرت نتائج الدراسة التشريحية لأمراض أنسجة المبايض للمجموعة CYP2 انخفاضا في أعداد الحويصلات الثانوية و حويصلات غراف مقارنة بالمجموعة المرجعية مع عدم اختلاف أعداد الحويصلات الأولية والأجسام الأصفر. كذلك، أظهرت الدراسة وجود احتقان في الأوعية الدموية في مقاطع تشريحية من المبايض لإناث من نفس المجموعة. أظهرت الدراسة أيضا أن أنسجة مبايض الإناث

التي تعرضت ل CYP2، SIG2 و MIX2 كونت العديد من الأنوية الصغيرة والفراغات الكبيرة بين الخلايا، كما ارتفع عدد الحويصلات الأتريتية Atretic follicles (51%) مقارنة بالمجموعة المرجعية (15.5%). بشكل عام، المبيدان سايبرميثرين وسيجنوم و مخاليطهما يؤديان الى العديد من التغيرات النسيجية لمبايض الفئران التي تعرضت للجرعات الأعلى (20 ملغ/كيلوغرام) من المبيدين.

في أنسجة المبايض للمواليد الإناث لم يكن هناك اختلاف في اعداد الحويصلات الأولية والثانوية بين جميع المجموعات. إلا أنه ظهر هناك انخفاض في عدد حويصلات غراف في المجموعة CYP2 (1.0) مقارنة بالمجموعة المرجعية (3.5). بالأضافة لذلك، فقد كان معدل العدد الكلي للحويصلات في المجموعة المرجعية أعلى من المجموعات الأخرى. هذا بالإضافة إلى انخفاض واضح في أعداد الأجسام الصفراء في مجموعة SIG2 (0.5) مقارنة بالمجموعة المرجعية (4.8). بشكل عام، فقد أظهرت المجموعات إنخفاضا بسيطا في نسبة الحويصلات الأتريتية مقارنة بالمجموعة المرجعية. وبالتالي فإن المبيدان المستخدمان بالجرعة الأعلى (20ملغ/كغم) يؤديان إلى خلل في أنسجة مبايض إناث الفئران المولودة للأناث التي تعرضت للمبيدان قبل وأثناء الحمل والرضاعة.

أما فيما يتعلق بالسمية الجينية، فقد كشفت نتائج فحص الRAPD على الDNA المستخلص من خلايا دم من الفئران قبل وبعد التعرض للمبيدان، وجود ما مجموعه 73 قطعة صغيرة من DNA متعددة الشكل (polymorphic bands) و تمثل تقريبا 25% من عدد القطع الإجمالي الناتجة. جميع المجموعات باستثناء المجموعة المرجعية كونت قطعا من المادة الوراثية متعددة الشكل تراوحت بين 6 (CYP1) و 22 (SIG1). نتائج حساب تشابه ال DNA قبل و بعد التجربة، تراوحت بين 94.28% (CYP1) إلى 77.92% (SIG1). وكان مؤشر التشابه في المجموعة MIX1 84.99%، في حين كان المؤشر 82.15% في المجموعة MIX2. معدل مؤشر التشابه بعد دمج الجرعتين 10 و 20 معا، كانت 90.31(CYP)، 84.91 (SIG) و 83.57 (MIX). تشير هذه النتائج إلى أن المبيدان المستخدمان يسببان سمية جينية في جميع الجرعات المستخدمة. بالإضافة إلى ذلك، فإن مزج المبيدين معا يزيد من السمية الجينية.

1. Introduction:

Chemicals are increasingly synthesized and used almost in every aspect of life around the Globe. Many of these chemicals are pollutants that can accumulate and harm humans and the environment. Chemical pollutants can enter the body through inhalation, food, water or skin. Pesticides are chemical compounds that are manufactured to control harmful pests in different aspects, especially agriculture. They are considered among the most heavily worldwide used chemicals that pollute the environment and endanger human and animal health. Pesticides can of natural origins (plant, animal and microbial origin) such as nicotine and rotenone (plant origin) or they can be synthetic such as DDT and organophosphorus esters. Synthetic pesticides were first used after World War II (Salem and Olajos, 1988; Garcia *et al*., 2012;). The first pesticide, DDT, was synthesized in 1874 (Garcia *et al*. 2012). For decades, manufacturing companies have been developing and enhancing different types of pesticides. In 2007, there were more than 1,055 active ingredients registered as pesticides (Goldman *et al*., 2007). These active ingredients yield over 20,000 pesticide products that are marketed in the United States (CDC, 2013). In 2019, approximately, 2 million tons of pesticides were utilized worldwide; however, by the year 2020, the global pesticide usage has been estimated to increase up to 3.5 million tons (Sharma *et al*., 2019). Pesticides can target a specific pest or can have a broad activity according to their chemical composition. Pesticides are classified according to the group of pests they target. They include herbicides, insecticides, fungicides, molluscicides, algaecides, and nematicides. Pesticides are crucial for sucessful cultivation of crops in order to prevent crops loss and meet the increasing demand for food production (WHO, 2018). Pesticides can also be used in public health activites especially in eleminating or controlling vector born diseases such as malaria (Garcia *et al*., 2012).

Pesticides are toxic to the environment, farmers and consumers. Their toxicity differs based on their target, chemical structure, exposure concentration and route. For example, insecticides are more toxic to human than herbicides (WHO, 2018). Pesticides can be extremely hazardous and causes serious health problems. Humans can be exposed to pesticides through, inhalation and skin, especially for workers and farmers. However, other people can be exposed to pesticides through ingesting its residues in food and water (WHO, 2010). The human health and environmental costs due to pesticides in the United States was estimated to be $9.6 billion/year (Pimentel, 2005). Pesticides are categorized into 3 different categories according to their toxicity level (Garcia *et al*., 2012):

1. Class I: Extremely Dangerous
2. Class II: Moderately Hazardous
3. Class III: Slightly Hazardous

Pyrethroids are one of the most used insecticides globally (Marettova *et al.*, 2017). They are synthetic neurotoxic pesticides that have been registered in the late 1970s and are based on the naturally occurring pyrethrins, which are found in the flowers of pyrethrum plant (*Chrysanthemum cinerafolis*) (Casida, 1980, Khambay & Jewess, 2005). Even though pyrethrins are found naturally and considered as the most effective natural insecticide, their instability in air and light makes them ineffective in agriculture (Casida, 1980). Therefore, pyrethroids were re-structured to increase the stability and insecticidal activity and decrease mammalian toxicity. After it had been registered, pyrethroids replaced other insecticides and it expanded rapidly. Pyrethroids can be used not only as an agricultural insecticide but also to control other insects that are vectors of diseases. Their insecticidal activity is mainly due to the ability to induce a prolonged neuron depolarization via alteration of sodium channels sensitivity in the nerve membrane by increasing sodium permeability and thus affecting nerve excitability (Soderlund, 2010; Chrustek A, 2018).

Pyrethroids are more toxic to insects than to mammals by about 2250 times; their use increased during the past 20 years and are considered as the fourth major group of insecticides (Chrustek A, 2018). Its residue has been found in soil, rivers, sediments and food (Sun *et al*., 2014).

Humans can be exposed to pyrethroids via several routs, and, thus have several effects on general health. Exposure via ingestion can be dangerous especially if it is ingested in relatively high amounts. The body can absorb up to 36% of ingested pyrethroid which then can lead to several problems based on the nature and the concentration of these pyrethroids (Ray *et al*., 2000). Once absorbed it can travel through the blood stream and can cross the blood brain barrier *(*Wakeling *et al*., 2012). Pyrethroids clearance from the blood is a fast process that can occur in a matter of hours; although some portion remain unmetabolized in fatty tissues where the $T_{1/2}$ ranges between 5 to 10 days after oral exposure (Marei *et al*., 1982).

Cypermethrin is a synthetic pyrethroid insecticide (Figure 1) that was first marketed in 1977 and affects the central nervous system (WHO, 1989). It's half-life in soil is about 30 days, although in foliage it's only about 5 days (NPIC, 1998). Based on a WHO report, its residues in food was considered low and ranged between 0.05 to 2.0 mg/kg and this amount is expected to be reduced during food processing (WHO, 1989). According to the same report, cypermethrin is rapidly absorbed in the gastrointestinal tract and is secreted with urine and feces.

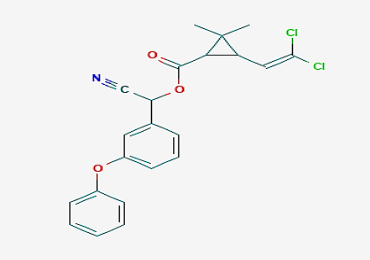

Figure 1: The chemical structure (2D) for cypermethrin (PubChem, 2005)

According to Crawford *et al*. (1981), the highest residues of Cypermethrin was found in fat tissues, it was also reported by Rhodes *et al*. (1984) that the elimination of Cypermethrin occur slowly in fats and skin. In a study on deltamethrin (pyrethroid insecticide), its residues were detected in blood plasma after 2 hours of oral administration and the concentration decreased with time. It was not detectable after 48 hours. As for the residues in fats, deltamethrin was detected and remained constant for days (Kim *et al*., 2008).

Signum is fungicide that is composed of two active ingredients: 26.7% Boscalid and 6.7% Pyraclostrobin. Boscalid belongs to Carboxamide while, Pyraclostrobin belongs to Strobilurin class (Callens *et al.*, 2005).

Strobilurins (Q_oI inhibitors), are a group of fungicides that have been isolated from fungi (basidiomycetes, genus: *Strobilurus*) (Vincelli, 2002; Balba, 2007). Strobilurin (strobilurin A and B) were first isolated by Anke *et al*. (1977). It was first commercially produced in 1996 and by 1999 strobilurin sales represented 10% of fungicides marketed globally (Bartlett *et al*., 2002). Strobilurin has β-methoxyacrylic acid that targets Q_o of cytochrome b complex III in the mitochondrial electron transport chain preventing energy synthesis by inhibiting electron transfer from cytochrome b to cytochrome c_1 (Bartlett *et al*., 2002; Nofiani *et al*., 2018). As almost all natural products, natural Strobilurin is unstable for agricultural uses as it breaks rapidly in sunlight (photodegradation), making its direct use in agriculture not feasible (Bartlett *et al*., 2002; Balba, 2007). Pyraclostrobin is one of the synthetic products that belongs to Strobilurin fungicides (Figure 2).

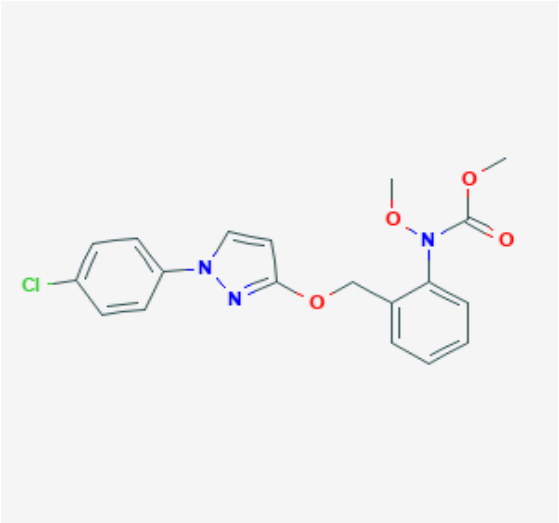

Figure 2: The chemical structure of pyraclostrobin (PubChem, 2006)

Studies on rats show that 50% of ingested pyraclostrobin is absorbed by the body with peaks appearing in the plasma within the first 30 minutes then 8 or 24 hours after exposure. After 2 days of exposure, 74-91% of ingested pyraclostrobin was excreted via faeces and (10-13%) via urine (Australian Pesticides and Veterinary Medicines Authority, 2003). In some experiments, rats and humans skin were exposed to different concentration of pyraclostrobin; 21-51% in rats and 3-8% in human of the doses were absorbed after 24 hour of exposure (Australian Pesticides and Veterinary Medicines Authority, 2003).

Boscalid (2-chloro-N-(4'-chlorobiphenyl-2-yl) nicotinamide) is a broad-spectrum carboxamide fungicide that has been registered in 2003. In soil, boscalid degrades slowly with low mobility (USEPA, 2003). Plants can take it through leaves and transport it through the xylem. It targets the electron transport chain in the mitochondria especially succinate dehydrogenase (SDH), and thus it is effective in controlling fungal spores germination (Stammler *et al*., 2008).

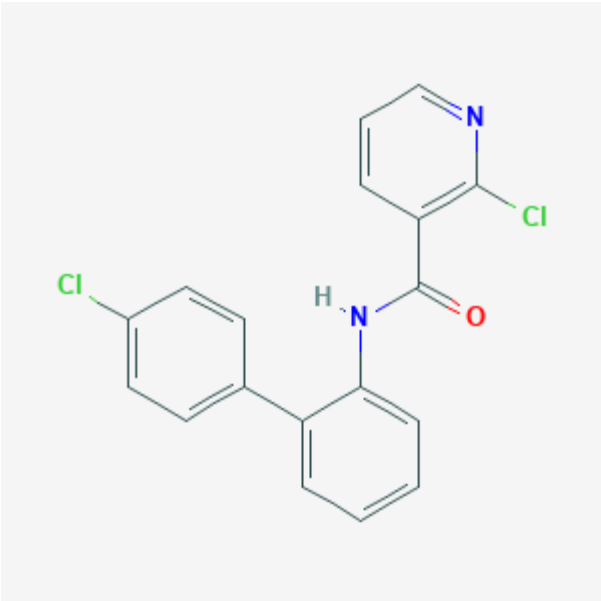

Figure 3: The chemical structure of boscalid (PubChem, 2005)

Residual study on cow shows that 1% of the total administered boscalid dose was found in milk and tissues and about 90% of the total dose was excreted (Australian Pesticides and Veterinary Medicines Authority, 2004).

A residual study (European and Australian trials) done on both pesticides (boscalid and pyraclostrobin), 40g/L (202g boscalid and 102g pyraclostrobin) were sprayed on apple trees; after 28 days residues of both pesticides were detected and found to be between 0.075-0.703mg/kg boscalid and 0.04-0.258mg/kg pyraclostrobin (Robinson, 2006).

Pesticides are toxic to living organisms as well as human beings and are associated with several diseases such as cancer, asthma, allergy, hormonal disturbance…etc. Exposure to pesticides can be due to direct contact by workers (farmers) or ingesting contaminated food or by indirect contamination from maternal exposure to pesticides (Kim, *et al*., 2017). Children are the most likely to be affected by pesticides because they are growing and their systems are not fully developed (Lozowicka, 2015). Pesticides effects can be either chronic or short term and can have significant effects on the body systems such as nervous system, immune system. Besides, they can

cause cancer and endocrine disruption (Damalas & Eleftherohorinos, 2011; Hu *et al*., 2015). According to Lozowicka (2015), it is more likely for children who were exposed periodically to pesticide to develop brain cancer than others. Pesticides also cause asthma-like symptoms. For example, in Norwegian population, it was found that the ratio of non-atopic to atopic asthma is greater in farmers than the non-farmers (Hoppin *et al.*, 2008).

Some pesticide are even considered genotoxic. Luaces et al. (2017) studied the genotoxic effect of the glyphosate Round Up on mammalian lymphocytes. They found that this pesticide causes chromosomal aberration at all used concentration and it caused a delay in the cell cycle. Another study showed that cypermethrin is a genotoxin that causes an increase in the sister chromatid exchange in mice bone marrow cells (Amer *et al.*, 1993). Xiong *et al*., (2015), found that pesticides, especially Fenpropathrin (pyrethroid), have an ability to degrade dopamine, a potential risk factor for Parkinson disease.

Pesticides can also cause a serious oxidative stress in the body that results in an increase in antioxidant enzymes in the blood (Prakasam *et al*., 2001). This is the reason behind the significant elevation in antioxidant enzymes among farmers (sprayers) compared to the control group (Prakasam *et al*., 2001). The study also found a higher concentration of thiobarbituric acid reactive substances in the sprayer farmers compared with the control group.

Pesticides can disrupt the endocrine function by either mimicking or blocking endocrine action. In addition, they can interfere with the receptors and hormones synthesis causing serious problems that can disrupt the reproductive system (Mnif *et al*., 2011; Schug *et al*., 2011). A recent study (Rattan *et al*., 2017), showed that females with DDT residues in their blood had early menopause. Moreover, they found that pesticides could reduce ovary weight and cause serious changes in the ovarian tissues.

Endocrine disruptors can have different effects on the reproductive hormones as well. They can either inhibit the production of some hormones such as estradiol and progesterone or compete with these hormones on their receptors (Schug *et al*., 2011). Besides, some pesticides can disrupt hormones expression in the hypothalamus or increase hormones production and activity *(Mnif et al.*, 2011).

As mentioned earlier, pesticides are heavily used around the World in different fields. Due to that, pesticides residues can be found in different food items such as water, milk, meat vegetables…etc. In a study from Spain, 16% of raw milk was found to be contaminated with Triazin (a widely used herbicide) (Castillo *et al.*, 2012). Lozowicka *et al.* (2014) reported that 10 active pesticides substances were detected in grain samples; some of which, such as DDT, are banned.

Pesticide residues can be found in the environment as well. Chaza *et al*., (2017) reported that pesticide residues were detected in 15 different groundwater samples, which were collected from Akkar (Lebanon). They found that organochlorine pesticides and DDT were detectable in 95-100% of the groundwater samples. According to Pan *et al*. (2017), organochlorine and organophosphorus pesticides residues were detected in soil and groundwater samples from the Yangtze River Basin (China).

2. Literature Review:

For decades, manufacturing companies have been developing and enhancing different types of pesticides. Pesticides can target a specific pest or can have a broad activity according to their chemical composition. Between 2010 and 2014, global mean annual pesticide use was 2.78 kg/ha (Zhang, 2018). Japan has the highest average annual pesticide use (Kg/ha) as 18.94 followed by China 10.45, Mexico 7.87, Brazil 6.166, Germany 5.123, France 4.859, UK 4.034, USA 3.886 and India 0.261 (Zhang, 2018). According to De *et al*., (2014), most pesticides used (47.5%) were belonging to herbicides category, 29.5% were insecticides, 17.5% were fungicides and 5.5% other pesticides.

Pesticides have diverse effects on health that can range from being carcinogenic to being allergens. Pesticides residues can be found in the environment as well as in tissues and blood of living organisms. Sharma *et al*., (2015) reported that pesticides residues were detected in 35% of human blood samples. Common impacts of pesticides on animal and environmental health vary from oxidative stress to toxicity and genotoxicity, reproductive impairment, embryo development disorders and endocrine disruption.

Giray *et al.* (2001) reported that both single (170 mg/kg) or repeated (75 mg/kg) oral administration of cypermethrin significantly cause oxidative stress in cerebral and hepatic tissues of rats. Another study on mice (Jin *et al*., 2011), found that 3 weeks (postnatal day 21-42) exposure to 20 and 10 mg/Kg to cypermethrin causes an increase in antioxidant enzymes activity as well as their encoding genes. According to Muthuviveganandavel *et al*., (2008), different doses of cypermethrin (5, 10, 25, 50 mM) cause an increase in Malondialdehyde (MDA), Alanine Transaminase (ALT), Alkaline Phosphatase (ALP) and Gamma Glutamyl Transferase (GGT) levels in different tissues and serum of mice.

Strobilurin (pyraclostrobin) has β-methoxyacrylic acid that target Q_o of cytochrome b complex III in the mitochondrial electron transport chain preventing energy synthesis by preventing electron transfer from cytochrome b to cytochrome c1 (Bartlett *et al.*, 2002; Nofiani *et al.*, 2018). Fang et al. (2016) reported that pyraclostrobin induces the activity of detoxifying enzymes such as glutathione S-transferase (GST) in typical water flea (Daphnia magna). Zhang et al. (2017) studied the toxicity of pyraclostrobin to zebra fish. They reported that Reactive Oxygen Species (ROS) levels were elevated in all treatments (0.001, 0.01 and 0.02 mg/l for 7, 14, 21 and 28 days) compared to the control. However, ROS levels slightly decreased, as the time of exposure increased, suggesting an antioxidant enzyme activity. Super Oxide Dismutase (SOD) activity decreased with an increase in pyraclostrobin concentration suggesting an inhibition of SOD activity due to the excess ROS. As for Catalase (CAT), its concentration was lower in all pyraclostrobin concentration at 7, 14 and 21 days of exposure. However, its concentration increased as pyraclostrobin concentration increased and it was even higher than the control in 0.02 mg/l for 28 days suggesting that CAT, to some extent, can detoxify ROS. GST levels increased in a dose dependent manner after it decreased in the first dose (0.001 mg/L) compared to the control. On the other hand, carboxyamide (boscalid) targets the electron transport chain in the mitochondria especially succinate dehydrogenase (SDH) (Stammler *et al*., 2008).

Some pesticides are classified as endocrine disruptors. In a study by Jin *et al*. (2011), testosterone levels in mice significantly decreased after 3 weeks of orally administered 20 mg/kg cypermethrin. Another study (Hu *et al*., 2011), showed that serum level of testosterone in rats was reduced after 15 days exposure to 50 mg/kg cypermethrin.

Female hormones are also affected by pesticides exposure where their concentrations can be either elevated or decreased. In a study about the effects of cypermethrin and methyl parathion mixtures on hormone levels and immune functions in Wistar rats (Liu *et al*., 2006), the level of FSH

increased to (5.59±1.067mlU/ml) in intermediate (1.8mg/kg bw) and (5.750±0.936mlU/ml) high dose (8mg/kg bw) group compared to the control (3.373±2.171mlU/ml); there was an increase in the estradiol levels from (8.435±7.344pg/ml) in the control group to (14.16±5.06pg/ml) in the intermediate group and to (13.700±4.161pg/ml) in the high dose group. There was no significant change in the level of LH in all groups . Zhou *et al*. (2018b) reported that exposure of mice to cypermethrin decreased the level of progesterone from 10.94 ng/ml in control group to 9.34, 8.19 and 6.82 ng/ml in 5, 10 and 20 mg/kg cypermethrin-exposed groups, respectively. In contrary, the concentration of estrogen (E_2) increased from 49.07 pg/ml in control to 55.32, 66.01, 80.50 pg/ml in 5, 10 and 20 mg/kg cypermethrin-exposed groups, respectively. The estrogen receptor ERα was also upregulated from 1.103 in control group to 1.543, 6.827, and 17.877 in 5, 10 and 20 mg/kg cypermethrin-exposed groups, respectively. Another study showed that the level of both estrogen and FSH increased, while the level of progesterone and LH decreased after exposure of mice to cypermethrin (Zhou *et al*., 2018a). According to Montoya *et al.* (2014), boscalid, which is one of the two active ingredients of signum, has an indirect effect on the thyroid hormones.

Pesticides can also alter organs by either changing their weight or their anatomy. Grewal *et al.* (2010) suggested that exposure to cypermethrin can alter the weight of body organs. They reported that repeated oral exposure to 5 and 20 mg/kg of cypermethrin for 30 days changed the weight of the heart, liver, brain, kidney and testis by either increasing or decreasing their weight. They also found that repeated exposure causes severe damage to the tissues of these organs like neural degeneration, hemorrhage and sloughing off renal epithelial cell in the convoluted tubules, glumulari shrinkage, necrosis in renal tubes, striation loss of the cardiac muscle, alveolar septa thickening in the lung and loss of follicular cells and oocytes in ovaries. Another study (Zhou *et al*., 2018a), also reported that with the exposure to cypermethrin, there was a loss in the weight of

uteri and ovaries. The same study also reported that there was a reduction in the number of the small follicles and medium follicles.

Sangha *et al*. (2013) reported that the weight of ovaries and uterine in female rat were decreased by 15.4% and 68.2%, respectively, after oral administration of cypermethrin (50 mg/kg). They also reported that even though the number of follicles didn't differ between treatment and control, the percentage of atresia of follicles was higher in the treated rats. After 2 weeks of exposure, the percentage of atresia of follicles was 41.3% in the treated group compared to the control, 31.5%. The atresia percentage after 4 weeks of exposure was 39.1% in the treated group while in the control it was 32.80%. The follicles diameter in different stages was reduced in the treated group compared to the control.

According to a study by Luz *et al*. (2018), exposure to pyraclostrobin caused accumulation of triglyceride, mitochondrial dysfunction and reduced expression of some genes such as *Glut-4, Pkm, Pfkl, Pfkm, Cpt-1b, Fasn, Acacα & Acacβ* suggesting a disruption of metabolism.

When a cortical neurons culture was exposed to 100 µM of pyraclostrobin for 24 h, a significant decrease in cells viability was observed. Pyraclostrobin is one of the most neurotoxic pesticides with a very low LC_{50} that can increase intracellular calcium concentration with a very strong mitochondrial membrane depolarization (Regueiro *et al*. 2015).

In aquatic life, pyraclostrobin was reported to causes embryo toxicity in *Daphnia magna* after 21-day exposure to 0.15, 0.3, 0.6, 1.2 and 2.4 µg/L (Fang *et al.*, 2016). Belden *et al*. (2010), also reported that pyraclostrobin leads to a 100% mortality in *Bufo cognatus* tadpoles in different concentration (15, 150 and 1500 µg/L) and a significant increase in mortality of juvenile *B. cognatus* in medium and high toxicity levels.

According to Barbara, *et al*. (2009) cypermethrin was detected in human milk in low concentration suggesting that its source was food and agricultural exposure.

Montoya *et al.* (2014), reported that exposure of rats to boscalid (1500 ppm) causes an increase in body weight after 28 days of exposure and causes a decrease in food consumption by day 14 (no significant difference between day 14 and 28).

Fetuses can be exposed to pesticides when mothers are exposed. Pesticides such as organophosphates can cross the placenta barrier changing the cholinergic system in the placenta and affecting placental maturation thus, affecting the fetus development (Ramon-Yusuf *et al*., 2017). Lactation is also a second way through which an infant can be exposed to pesticides, Different studies suggested that mothers' milk can be polluted with pesticides residues, and thus increasing the exposure incident to the infants. Vall *et al*. (2014) reported that 34 (47.2%) out of 72 human breast milk samples were polluted with DDT residues (average: 0.92 ng/g) with a range between 0.08-16.96 ng/g. Bouwman *et al*. (2006) reported that pesticides (pyrethroids) residues were detected in 152 breast milk samples with the following concentrations (µg/L) permethrin (14.51), cyfluthrin (41.74), cypermethrin (4.24), deltamethrin (8.39) and Sigmapyrethroid (31.5). According to Taylor *et al*. (2009), cypermethrin was detected in human milk in low concentration suggesting that its source was food and agricultural exposure.

Wang *et al.* (2011) reported that the testes weight and spermatogenic cells layers decreased in pups after being exposed during lactation via exposing their mothers to 25 mg/kg cypermethrin. Singh *et al.* (2017) suggested that parental exposure of cypermethrin have long-term effect on reproductive function of F_1 that were then transmitted to F_2.

Pesticides can have serious effects on reproduction. According to Zhou *et al*. (2018b), the percentage of successful embryo implantation sites decreased in mice groups exposed to 10 and 20 mg/kg β-cypermethrin from 100% in control group to 40% and 30% in 10 and 20 mg/kg cypermethrin-subjected groups, respectively. Another study by Al-hamdani and Yajurvedi (2017),

confirmed these findings and reported that the rate of the successful pregnancy in mice significantly decreased after exposure to 2.76 and 5.52 mg/kg.bw.d β-cypermethrin to 80% and 60%, respectively compared to the control. Besides, the number of implantation sites decreased significantly in 2.76 and 5.52 mg/kg.bw.d exposed groups. Hartman *et al.* (2014), reported that the active ingredient of pyraclostrobin increased the developmental rate of tadpoles for 5 days when they were exposed to 1.7 µg/l compared to the control. Pyraclostrobin was tested on *Xenopus tropicalis* (western clawed frog) embryos; results indicated that, pyraclostrobin (0.5-6µg/l) reduced embryo survival and increased the percentage of malformation (Wu *et al.*, 2018).

Pesticides can be genotoxic. According to Amer *et al*, (1993), there was a dose-dependent increase in the frequency of sister chromatids exchange when bone marrow cells were exposed to 300 mg/kg cypermethrin (11.12 ± 0.05) compared to solvent and control groups (3.7 ± 0.14 and 4.4 ± 0.26, respectively) (Amer *et al.*, 1993). In mice spleen cells exposed to 4.00 µg/ml cypermethrin, sister chromatids exchange reached 15.1 ± 0.05 compared to solvent and control groups (8.6 ± 0.23 and 5.9 ± 0.39, respectively. Patel *et al.* (2006) also reported that there was a dose-dependent increase in DNA damage in several organs of mice (spleen, kidney, liver, bone marrow, lymphocytes and brain) subjected to cypermethrin. Kocaman & Topaktaş (2009), also confirmed the genotoxicity of cypermethrin where they reported that the sister chromatid exchange and chromosomal aberrations were significantly induced in lymphocyte cells treated with 5, 10, 15 and 20 µg/ml cypermethrin for 24 and 48 h. Micronucleus formation was significantly induced at 5 and 10 µg/ml of cypermethrin. Cayir, *et al.* (2014) reported that micronuclei formation in human lymphocytes increased significantly at doses 2, 6, 25 µg/ml Signum. In addition, the nucleoplasmic bridges significantly increased at a dose of 0.25 µg/ml pyraclostrobin. According to Zhang *et al.* (2017), pyraclostrobin is also genotoxic to zebra-fish. They reported that there was a significant dose dependent increase in DNA damage in exposed fishes.

In Palestine, various types of pesticides have been extensively used in agriculture for decades. The annual rate of use of pesticides in the West Bank was 502.7 tons in 2010 and 14 out of 123 of the used pesticides are internationally banned. In Gaza, about 893 tons were used during the year 2010 (PCBS 2010). 1348.14 tons/ hectare is the annual use in West Bank and Gaza in 2017 (WorldMeter, 2021).

Monitoring of pesticide residues in fruits and vegetables are lacking and awareness to risks associated with heavy, unsafe use of pesticides is minimal. Furthermore, the concept of organic farming is almost lacking. Farmers usually use a mixture of an insecticide and a fungicide to control pests in their farms. This situation necessitates establishing studies and campaigns that investigate the negative health impacts of pesticides and their residues. Therefore, this study aims at evaluating the negative impacts of two common pesticides, the insecticide Cypermethrin & the fungicide Signum, on reproductive biology and DNA integrity of albino rats and their offspring.

Studies on Cypermethrin are numerous while the studies on Signum fungicide effects on human or mammalian health is scarce. There is no studies on the effect of combined exposure of both pesticides. The present study aims to evaluate the effect of single exposure to different pesticides (Cypermethrin and Signum) and their mixture on the reproductive biology of female albino rats and their potential Genotoxicity.

3. Materials and Methods:

3.1 Pesticides

Pesticides Cypermethrin and Signum were obtained from local stores in Palestine.

3.2 Experimental setup

A total of 31 female Sprague Dawley albino rats (140-200 g body weight) were obtained from the Animal Unit of the Dept. of Biology and Biochemistry, Birzeit University. They were subdivided into 7 groups (control, 10 mg/kg bw cypermethrin, 20 mg/kg bw cypermethrin, 10 mg/kg bw signum, 20 mg/kg bw signum, a mixture of 10 mg/kg bw cypermethrin and 10 mg/kg bw signum and a mixture of 20 mg/kg bw cypermethrin and 20 mg/kg bw signum) and were kept in standard animal cages **(Table 1)**. They were provided with standard diet and water *ad libitum* throughout the study. Blood samples were collected prior the experiment and were placed in EDTA tubes for later genotoxicity testing. Female rats were allowed to mate from the first day of exposure, and males were left with females in the cages to birth.

Table 1: Summary of the experimental setup.

Group #	Treatment	Code	No. of female rats	Mean weight ± SEM (g)
1	control	C	4	152.33±5.18
2	10mg/kg bw cypermethrin	CYP1	4	161.25±2.96
3	20mg/kg bw cypermethrin	CYP2	5	151.4±2.25
4	10mg/kg bw signum	SIG1	4	154.75±6.51
5	20mg/kg bw signum	SIG2	5	166.8±2.67
6	10mg/kg bw cypermethrin + 10mg/kg bw signum	MIX1	5	179±1.12
7	20mg/kg bw cypermethrin + 20mg/kg bw signum	MIX2	4	179.5±0.29

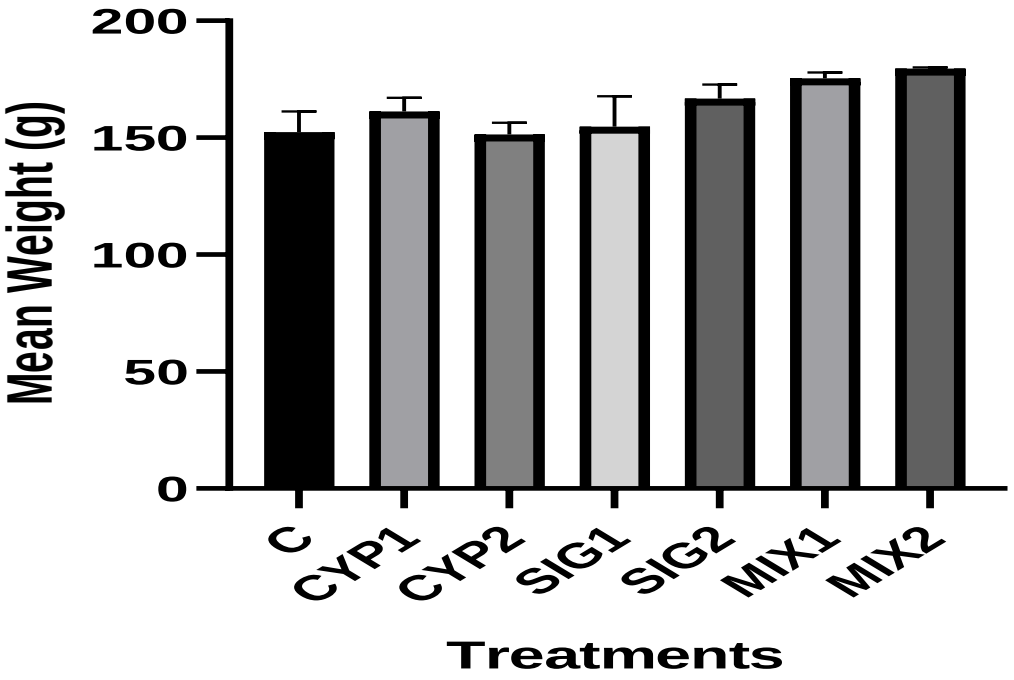

Figure 4. Mean weights of female rats in each group at the beginning of the experiment. Error bars represent standard error of mean (SEM).

Each female in the 7 groups was given 0.5 ml of the appropriate treatment by gavage 5 days a week. Female rats were subjected to treatments from day one of the experiment to day 21 after birth (42 days in total). The females were monitored and their weights were recorded on a weekly basis throughout the experiment. After birth, offspring number, sex and weight (weekly up to 3 weeks) were recorded. Blood samples were collected from both mothers and female offspring after weaning (21-24 days after birth) and were kept in EDTA tubes for later genotoxicity testing (mothers) and pesticide residues analysis (offspring). Other blood samples were kept in plain tubes for serum preparation and hormonal testing. Finally, both mothers and female offspring were sacrificed and their ovaries were obtained and stored in 10% formalin for histopathological analysis. Blood samples for genotoxicity testing were stored at -32℃, while all other blood samples were kept at -80℃.

Two females from MIX1 and 1 female from SIG1 groups get pregnant for the second time after they gave birth to the first pups. During the second pregnancy and lactation periods, females continued to receive the designated treatment. After birth, pup's weights were recorded on weekly basis for 21 days.

3.3 Genotoxicity Testing:

DNA was extracted from 14 blood samples (2 rats/ group) according to the protocol provided by DNA extraction kit (AccuPrep® genomic DNA Extraction Kit, Bioneer Corporation, Republic of Korea). Thirteen decamer primers (Hylabs, Hy Laboratories Ltd), were used in this study (Table 2). The PCR reaction volume was 20µl using a ready PCR mix (Bioneer Corporation, Republic of Korea), 3µl of DNA, 5µM primer were added to each PCR tube; nuclease free water was added for a final volume of 20 µl. The ladder (Marker) used was GeneDirexÒ 100 bp DNA Ladder RTU & GeneDirexÒ 100 bp DNA Ladder H3 RTU.

The PCR mixtures were subjected to the following PCR program: initial denaturation for 3 minutes at 95^{o}C, followed by 41 cycles of 30 sec at 95 ^{o}C, 30 sec at 35 ^{o}C, and 40 sec at 72 ^{o}C in a thermocycler (T100 Thermal Cycler Bio-Rad Laboratories, Singapore). PCR products were resolved on 1.5% agarose gels that were stained with ethidium bromide (Sigma, St. Louis, MO). The DNA were then exposed to UV light and were documented using a UVITEC gel documentation system (Cambridge, UK). In order to check for the repeatability and the integrity of the DNA, a reproducibility test was done with different primers and different groups including the control.

Table 2: Primers used in this study

#	Primer	Sequence 5`-3`	G-C content (%)
1	A04	AAT CGG GCT G	60
2	C1	TGC GCC CTT C	70
3	C06	GAA CGG ACT C	60
4	PG3	GCA TGC GAT C	60
5	A03	AGT CAG CCA C	60
6	APO8	ATG CAG GCT T	50
7	B9	GTT TCG CTC C	60
8	PA09	TCT GCT CTC C	60
9	PA02	GAC CAT TGC C	60
10	04	AGG GCC CGG G	90
11	PG5	TTC GAC CCA G	60
12	PG9	GCT GCT CGA G	70
13	PG12	CCA GCC GAA C	70

3.4 Similarity Index:

DNA similarities before and after the treatments were calculated according to Nei & Li (1979):

$S_{XY} = 2n_{xy}/n_x + n_y$

Where, $S_{XY,}$ is the similarity index between two organisms (before and after the treatments in this experiment); n_{xy}, is the number of common bands before and after the experiment; n_x. is the total number of bands before the experiment; n_y, is the total number of bands after the experiment ended.

3.5 Serum Hormonal Levels:

Blood samples were centrifuged within 15 minutes of blood collection at 4000 rpm for 10 minutes. Serum samples were then separated into new Eppendorf tubes and stored at -80℃ until analysis. Two to three samples were analyzed in duplicate for all four hormones tested (LH, FSH, Progesterone and Estrogen) via purchased ELISA kits.

Serum estradiol levels were determined using Estradiol Rat ELISA Kit (Demeditec Diagnostics GmbH, Germany) as follows: 75µl of calibrators and samples were dispensed in separated wells as duplicates; 50 µl of the incubation buffer was dispensed into each well and then were incubated at room temperature for 2 hours on an Orbital Shaker (IKA™ KS 260basic); the enzyme conjugates were diluted 1:100 in an enzyme conjugate diluent and 50 µl was dispensed into each well. Thereafter, it was incubated at room temperature for 1 hour on a plate shaker; the content of teach well was discarded and the wells were then washed 4 times with diluted wash solution; 200 µl of substrate solution was added to each well followed by dark incubation for 30 minutes. After that, the reaction was stopped by adding 50 µl of stop solution to each well. Finally, the absorbance was determined at 450nm using a Unilab Microplate Reader 6000 (Model RTC 6000, USA).

The protocol of the Progesterone "Rat/Mouse" ELISA Kit (Demeditec Diagnostics GmbH, Germany) was followed in order to determine the serum progesterone level. 10 µl of calibrator and samples were dispensed into separated wells in duplicates followed by dispensing 50 µl on incubation buffer, enzyme conjugate and incubation for 1 hour at room temperature on an Orbital Shaker (IKA™ KS 260basic); the content of the wells were discarded and the wells were rinsed 4 times with diluted wash solution; 200 µl of substrate solution was added to each well followed by dark incubation for 30 minutes; the reaction was then stopped by adding 50 µl of stop solution and the absorbance was determined at 450nm via the Microplate Reader 6000.

For LH level determination, the serum samples were diluted 2x with sample dilution buffer and with 2.5x of assay buffer as recommended by the LH Rat (S-type) ELISA Kit manufacturer (DRG Instruments GmbH, Germany). The evaluation of LH serum level was then done as recommended by the manufacturer as follows: the coated plate was washed 4 times by washing buffer; 50 µl of samples and standard solution were added into separated wells and then were shaken on an orbital shaker for 30 second followed by 2hours incubation at room temperature. After that, the content of the wells was then discarded and the wells were rinsed 4 times with the washing buffer; 50 µl of biotin-labeled anti-LH solution was added to each well before being shaken on an orbit shaker for 30 seconds followed by incubation for 1 hour at room temperature. Thereafter, the content was then discarded and the wells were rinsed with washing solution followed by the addition of 50 µl of HRP conjugate avidin solution and were shaken for 30 seconds; the plate was then incubated for 30 minutes at room temperature; the reaction mixture was discarded; the wells were rinsed and the 50 µl of chromogenic substrate was added into each well and the plate was shaken and then incubated for 20 minutes at room temperature; the reaction was stopped by the addition of 50 µl of the reaction stopper to all wells followed by absorbance determination at 450 on the Microplate Reader 6000.

The evaluation of FSH level was done according to the protocol recommended by the FSH HS ELISA Kit manufacturer (DRG Instruments GmbH, Germany). 100 µl of Buffer 13, standards and samples were dispensed into separated wells followed by incubation on plate shaker for 30 minutes at room temperature; the wells were then washed 4 times with wash buffer; 100 µl of reconstituted FSH detector antibody was added into all wells except for the blank and the NSB (Non-Specific Binding) wells; 100 µl of assay buffer 13 was added to NSB wells. The plate was sealed and incubated at room temperature on a plate shaker for 30 minutes; the wells were washed 4 times with washing buffer followed by the addition of 100 µl of SA-HRP (Streptavidin conjugated to

Horseradish peroxidase) into each well; the plate was incubated at room temperature on a plate shaker for 30 minutes followed by washing 4 times with washing buffer; 100 µl of TMB (tetramethylbenzidine) substrate was added into each well and were incubated on a plate shaker for 30 minutes; the reaction was stopped by the addition of 100 µl of the stop solution into each well and read at 450nm via microplate reader 6000

The concentrations for all 4 hormones were calculated by interpolation (4PL) and with R^2= 0.99 via GraphPad Prism Software **(Fig. 1.A).**

3.6 Histopathology

At the end of the experiment, ovaries from the Control, CYP2, SIG2 and MIX2 groups were obtained and fixed in 10% formalin. Tissue samples were then dehydrated through a series of increased ethanol concentration (0-100%). They were then embedded in paraffin blocks. The paraffin blocks were sectioned and mounted, 0.5 mm section were deparaffinized and then rehydrated with a series of decreased ethanol concentration (100-0%). The sections were then stained with hematoxylin and eosin dyes. Finally, sections were examined under a light microscope (Leica, Germany). The examined samples were then photographed via Moticam X camera (Motic ®, USA).

3.7 Residues analysis

Blood samples collected from the offspring in EDTA tubes for residual analysis were stored at -80 °C until later analysis. In order to extract pesticides residues, 0.5ml of MQ, 1.5 ml CAN, 0.5g $MgSO_4$, 0.1g Na acetate and 50mg PSA were added to 0.5ml blood. 5µl of the extraction solution was injected in UHPLC (ACQUITY Arc, USA) column (C18, 3.5µm and (2.1mm*50mm)). Mobile phase is acetonitrile. **Figure 2.A** shows the Standard curve for boscalid (A) and pyraclostrobin (B).

3.8 Statistical Analysis:

All of the data were expressed as MEAN± Standard Error of Mean (SEM). The data were analyzed by GraphPad Prism8. One way ANOVA was used to compare between means of acquired data, a t-Test was done to compare between means of the first and the second pregnancy of group four, and the percentage of atretic follicle. $P<0.05$ were considered significantly different.

4. Results and Discussion

The exposure of rats to treatments continued for about 42 days. During this period, rats were carefully monitored and weights of females and pups were recorded on weekly basis. The appearance of white fur was used as indication of one week old pups while, opening eyes was considered as 14 days old. Results are summarized in the following sections.

4.1 Effect of CYP & SIG on Body Weight and Litter Size:

The percentage of conceived females in the groups was between 75% and 100% (**Table 3**). The percentage of pregnancy was 100% in all groups except the control and CYP1. This shows no impact of the treatments on pregnancy percentage under the experiment conditions.

Table 3: The percentage of conceived female rats in each group.

Group #	Treatment	No. of females in the group	Number of pregnant females	% of pregnant females
1	C	4	3	75
2	CYP1	4	3	75
3	CYP2	5	5	100
4	SIG1	4	4	100
5	SIG2	5	5	100
6	MIX1	5	5	100
7	MIX2	4	4	100

Weights of female rats were recorded over the 42 days of the experiment (gestation & lactation) and are summarized in **Table 1 A** in the appendix. During the whole experiment, no significant difference was observed between the weights of female rats in all treatments compared to the control group. Singh et al. (2017) reported that exposure of 3 months old female rats to 25% cypermethrin for 2 and 4 weeks did not cause a significant reduction in their weights. Liu et al.

(2006) also reported no significant difference in weights of female rats subjected to Cypermethrin and methyl parathion for 30 days. These findings are in agreement with the present study.

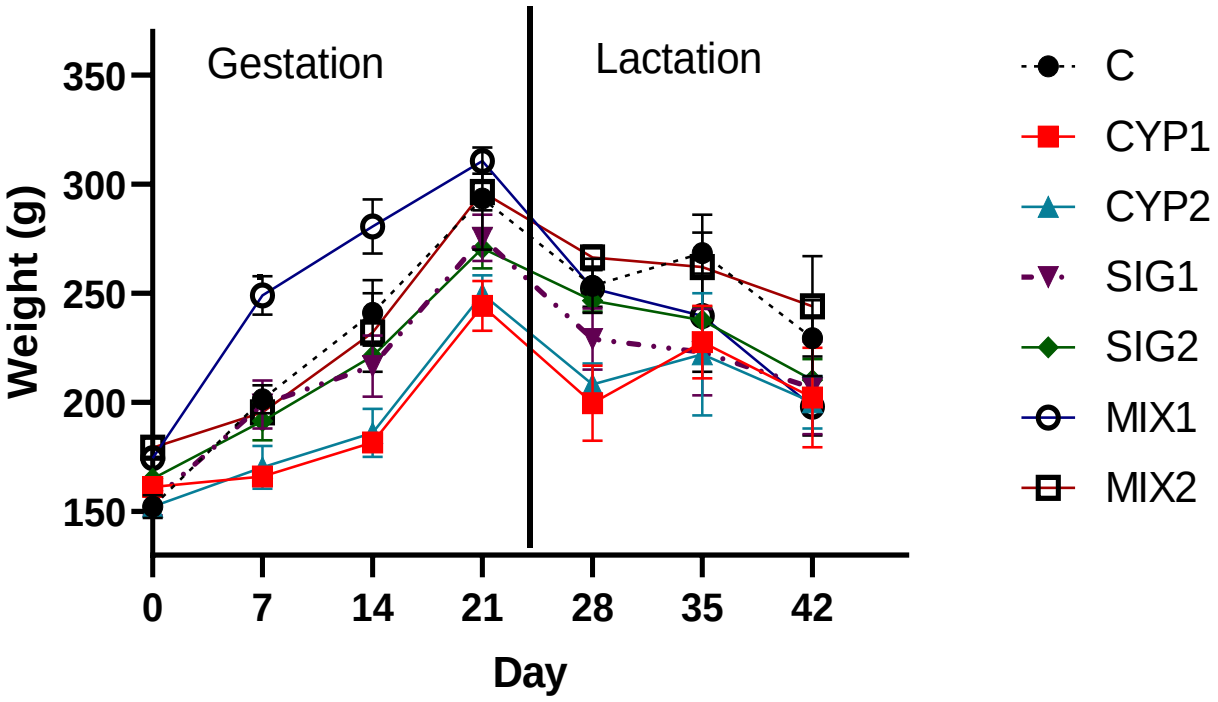

Figure 5: Weights (g) of female rats during pregnancy and lactation. The vertical line divides the two stages (gestation and lactation). Values represent mean± SEM.

When weights of female rats receiving the two doses of each treatment were combined together (CYP1+CYP2; SIG1+SIG2 and MIX1+MIX2) and compared to the control group on day 42 of the experiment, no significant differences in mean weights were observed (**Fig. 6**). This might indicate no significant effect of the two pesticides or their combination on weight of adult female rats.

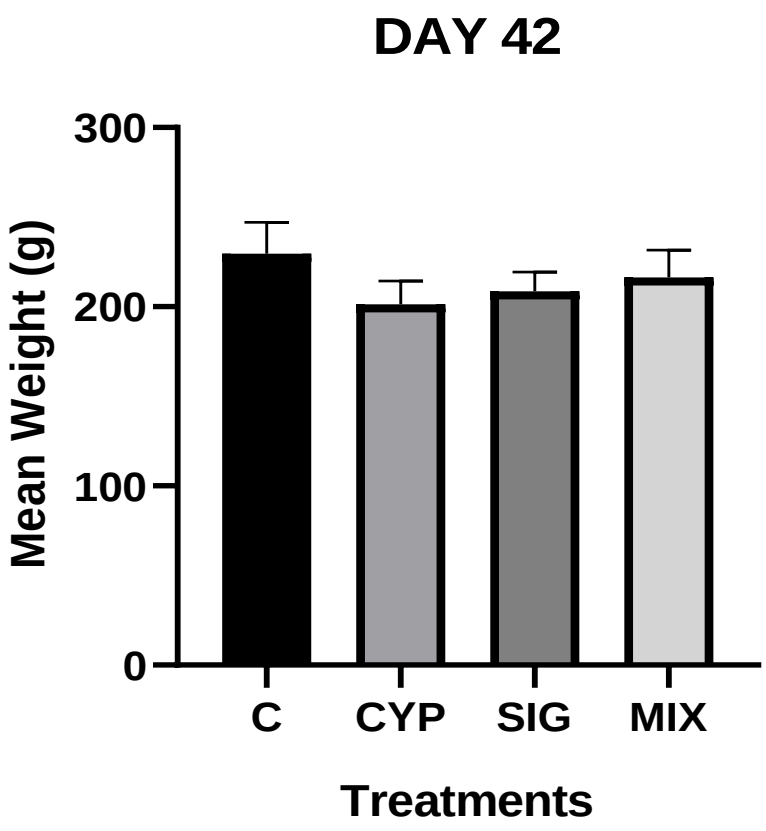

Figure 6: Mean weights of females receiving the two doses of the same treatment combined together on day 42 of the experiment. Values represent means±SEM.

In addition to weight, weekly change in weight was calculated per group (**Table 2.A**) and plotted in **Fig. 7**. It indicates also no significant difference between the control and the treatment groups with regard to weight change.

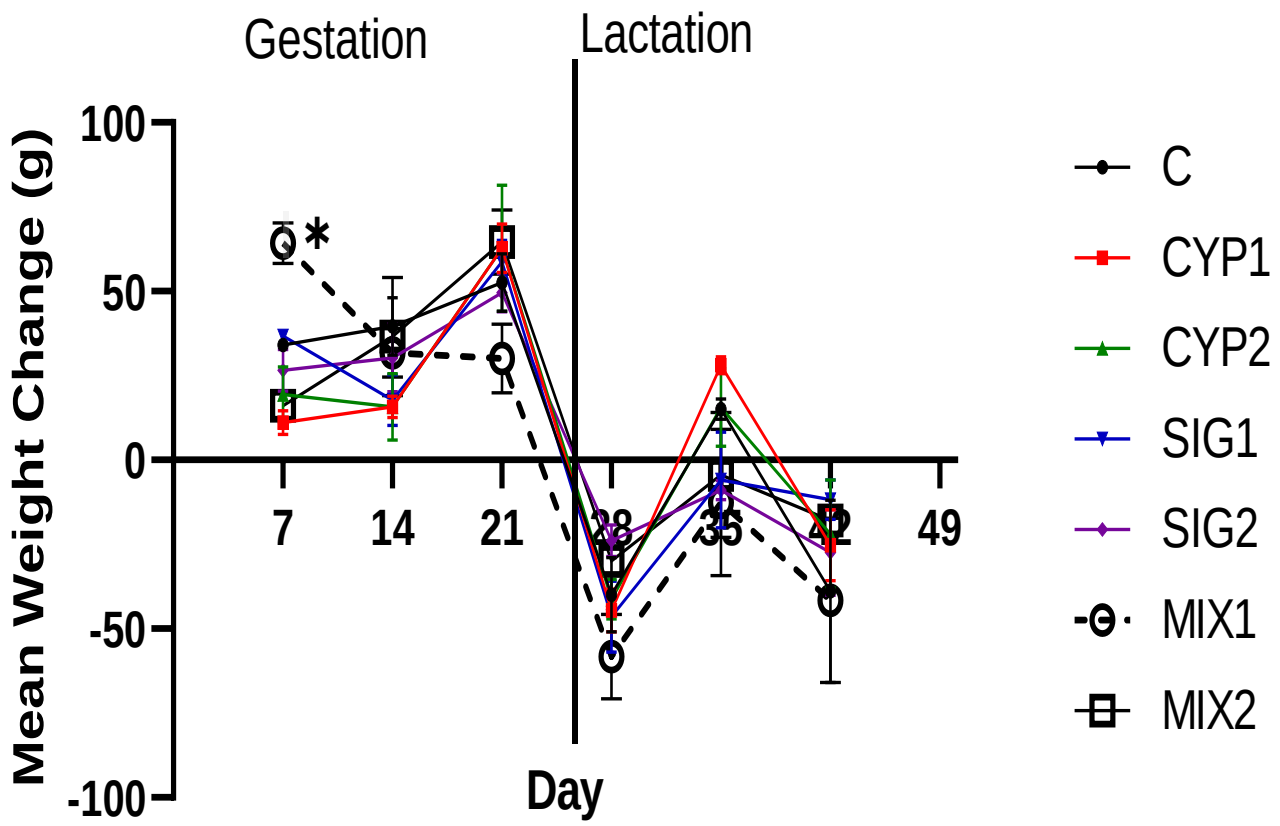

Figure 7: Weight change (g) in female rats during pregnancy and lactation.

Number of offspring/female and weight of pups/group at birth were recorded and the means were calculated and summarized in **Table 4**. No significant difference in neither mean number of offspring nor mean weight of pups was observed. This indicates no effect of CYP and SIG and their mixtures on both parameters during the experiment.

Table 4: The mean number of offspring per female in each group and mean weight (g) of offspring/group at birth

Group #	Treatment	Mean number of offspring/female	Mean weight of offspring/group (g)
1	C	8.0	6.2±0.6
2	CYP1	8.7	5.3±0.4
3	CYP2	8.8	5.2±0.4
4	SIG1	8.2	5.9±0.6
5	SIG2	7.0	7.2±0.4
6	MIX1	6.0	6.6±0.8
7	MIX2	7.0	6.0±0.2

From birth on, pups' weight was recorded on a weekly basis up to 3 weeks (day 21). Average weights of pups from each group were calculated and are summarized in **Table 3.A**. At birth, there was no significant difference in the average weight between the control group and other groups. Differences in average weight between the control and all other groups continued to be statistically insignificant at days 7 and 14. At day 21, there was a slight significant difference (P=0.023) between the average weight of MIX1 group (26.3±2.8g) compared to the control group (50.5±0.7g) (**Figure 8**). The mean weight of MIX2 (37.5±4.1) was less than the control group but with no statistical significance. However, death of weak pups between weekly weight recordings might has obscured some weight differences between pups of different groups.

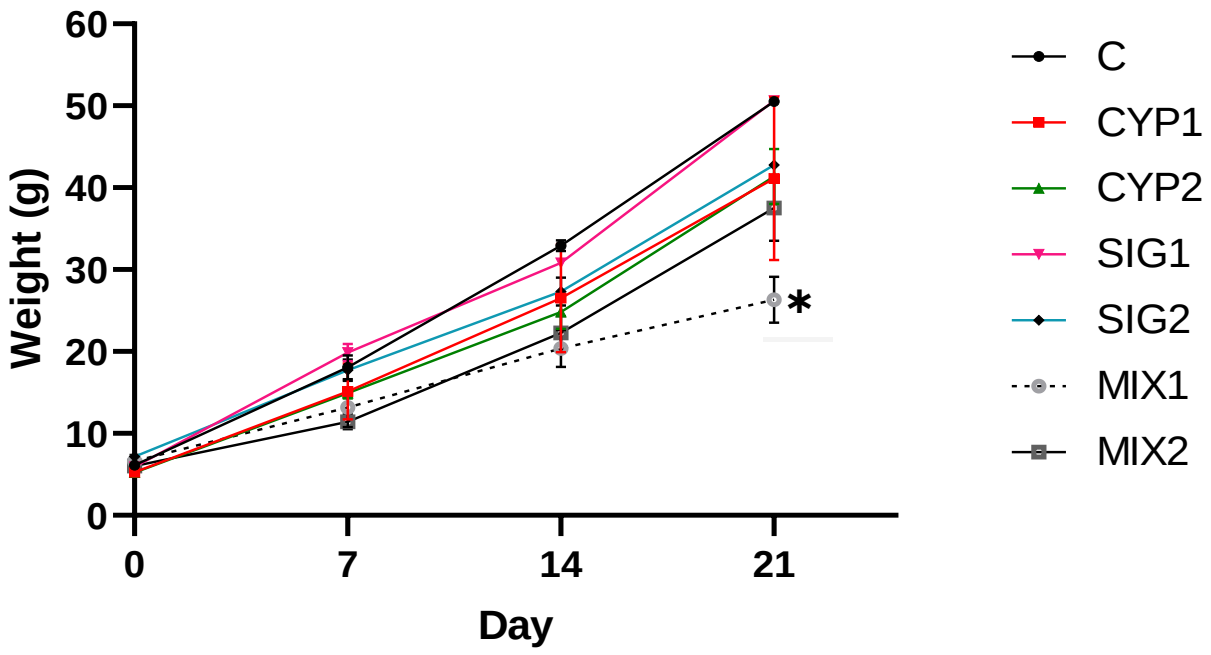

Figure 8: Pups weight (g) from birth (day 0) to weaning (day 21). Values represent mean± SEM. (*): indicates significant difference at P <0.05.

When combining weights of pups from groups that received the two doses of each pesticide treatment together (CYP1+CYP2; SIG1+SIG2 and MIX1+MIX2), and comparing them to the control, the following weight decreasing order (g) was observed: Control (50.50)>SIG (46.73)>CYP (41.29)>MIX (30.83) (**Fig. 9**). Statistical analysis shows that mean weights of pups of the control (50.5g) and SIG (46.73g) groups were significantly higher than those of MIX (30.83g) (P=0.022 and 0.021 respectively). CYP pups did not show any statistical difference in their weights from other treatments. This indicates the synergistic effect of combining the 2 pesticides together on weight of pups.

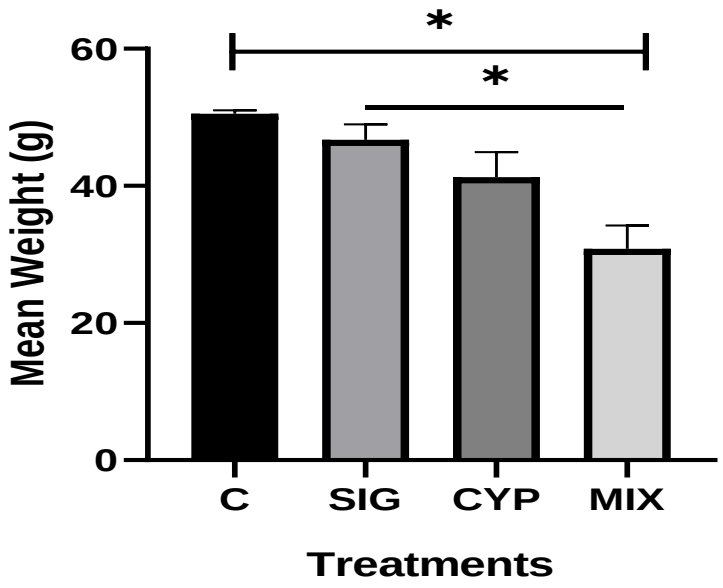

Figure 9: Mean weights of pups at day 42 after combining the 2 doses of each similar treatments. (*) indicates significant difference at P <0.05.

The findings of CYP are in agreement with the those of Ramon-Yusuf *et al*. (2017) who reported that there was no significant difference in the litter weight of Cypermethrin and dimethoate treated mice compared to control. Wang *et al.* (2011) also reported that maternal exposure of mice to 25mg/kg Cypermethrin has little to no effect on the pups weight. Hocine *et al.* (2016) reported that there was no statistically difference in pups' body weight between 0.02mg/kg alpha Cypermethrin treated group of rats and the control group. Lee *et al*. (2015) also reported that there was no difference between groups of mice treated with 0.1 and 0.2 mg/kg Cypermethrin and control groups. Singh *et al*. (2017) reported that there was no statistically difference in pups' body weight between groups of rats treated with Cypermethrin and the control. However, some other studies reported a decrease in pups weight from postnatal day 4 (PND4) to postnatal day 22 (PND22) in groups treated with 1mg/kg and 25 mg/kg Cypermethrin (Singh *et al*. 2017). Farag *et al.* (2007) also reported that maternal exposure to 10mg/kg body weight Cypermethrin lead to decrease pups

body weight from PND0 to PND 26. In the present study, there was a slight, but statistically insignificant decrease in pups that received CYP.

Studies on the effect of Signum on weight of animals are lacking. In the present study, although MIX1 pups showed statistically significant reduction in the weight at PND21, pups of MIX2 (higher dose than MIX1) showed no significant decrease. This makes the effect of both pesticides on offspring body weight inconclusive. Again, death of weak pups between weekly weight recordings might has obscured possible trends in weight differences between pups of different groups.

Two females from MIX1 and 1 female from SIG1 groups get pregnant for the second time after they gave birth to the first pups. All pups from MIX1 females died within the first week of birth, while most those from SIG1 (10/16) survived to reach weaning (21 days of age). During the second pregnancy and lactation, females continued to receive the designated treatment. Two tailed t-test was performed to investigate possible differences between pups' weights from the first pregnancy and the second one. Results indicated no significant difference between the pups' mean weight from the first pregnancy and the second pregnancy at birth (day 1) and at day 7 (**Fig.10**). Although mean weight of second pregnancy pups starts to show less increase than those of the first pregnancy. On day 14, the mean weight of pups from the second pregnancy (17.8 g) started to be significantly less (P=0.027) than those from the first one (30.82 g). This statistically significant difference continued over the third week (P=0.016) where mean weight of second pregnancy pups was 27g compared to 50.65g from the first one (**Fig. 10**). This might suggest a progressive impact of SIG on weight of rat pups over a prolonged maternal exposure during pregnancy and lactation.

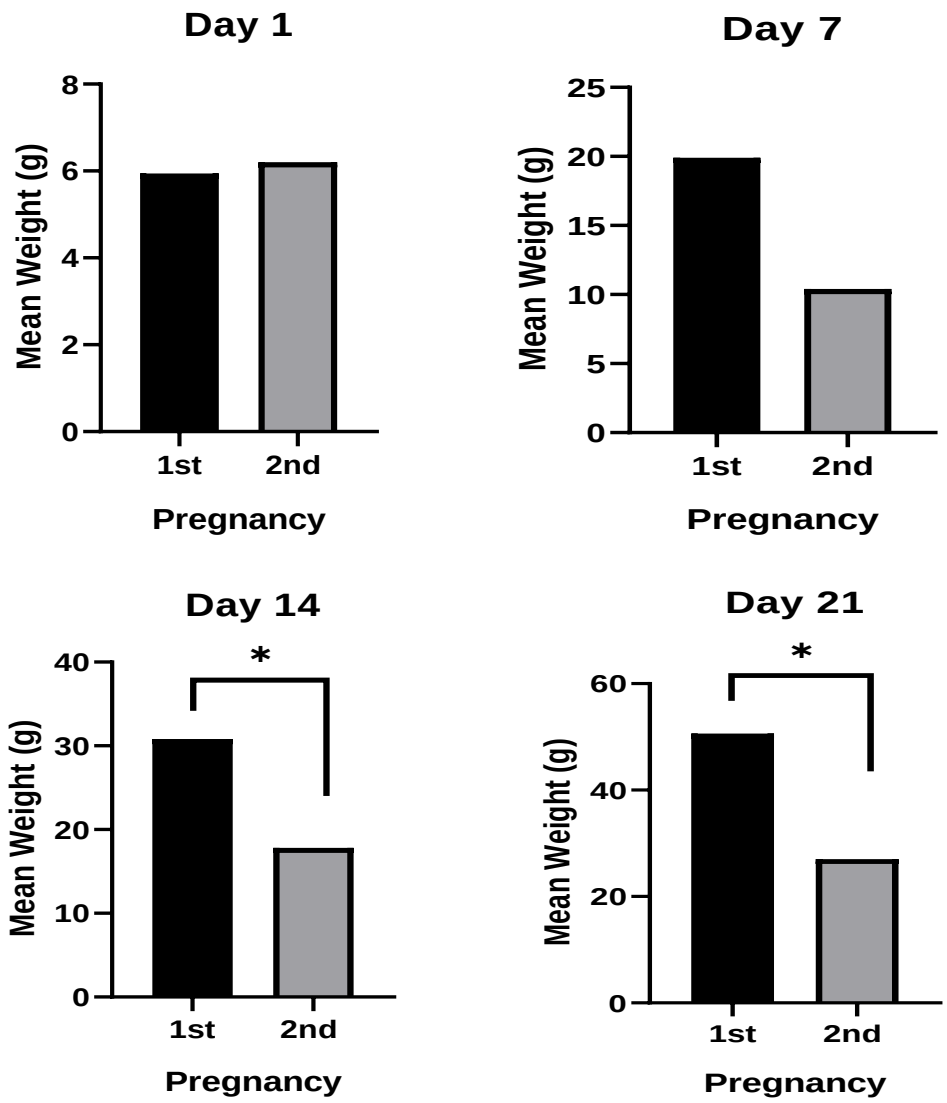

Figure 10: Comparison between pups' weight (g) from first pregnancy to those from the second one for treatment SIG1.

4.2 Effect of Pesticides on Serum Hormones Concentration:

As pesticides are known as endocrine disruptors, levels of four reproductive hormones were measured in female rats that were subjected to different pesticide treatments and in their offspring subjected to treatments via their mothers during pregnancy and lactation. These hormones were progesterone, estradiol, LH & FSH.

Progesterone is a steroid sex hormone that is involved in the menstrual cycle, pregnancy and embryogenesis. It is produced by the corpus luteum and prepares the uterus for pregnancy and thickening the uterine lining (Dante *et al*. , 2013).

Estradiol is an estrogen steroid hormone that is considered as the major female sex hormone. It is involved in the regulation of the estrous and menstrual female reproductive cycles. Estrogen stimulates granulosa cells proliferation and gonadotropin receptor level and it initiates embryonic implantation (Da Silva, 2010; Dey *et al.* 2015). Thus decreasing its concentration leads to fertility issues and might affect implantation (Zhou *et al.* 2018b).

Luteinizing hormone (LH) is a hormone produced by gonadotropic cells in the anterior pituitary gland. In females, it triggers ovulation and development of the corpus luteum. It acts synergistically with follicle-stimulating hormone (FSH). LH causes graafian follicle to rupture releasing oocyte ready to be fertilized (Da Silva, 2010). Thus decreasing their concentration leads to fertility issues and might affect implantation (Zhou *et al.* 2018b).

FSH (follicle stimulating hormone) is a gonadotropin hormone that is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland. It controls follicles growth and development (Hunter *et al*. 2004).

Table 4.A shows levels of the four hormones in blood samples collected from female rats after being subjected to pesticides for 42 days. No statistically significant differences in progesterone

and FSH levels were observed between groups of females subjected to pesticides and the control group **(Fig. 11)**. Whereas significant differences were observed in levels of estradiol and LH. Level of estradiol in CYP2 group was significantly higher than that of the C, CYP1, MIX1 and MIX2 groups (P=0.024, 0.016, 0.049 and 0.003, respectively). Signum groups (SIG1 & SIG2) did not show any significant difference neither from the C nor from CYP or MIX groups **(Fig. 11)**. This indicates that only the higher dose of CYP (CYP2: 20mg/kg bw CYP) caused a significant increase in estradiol. Both doses of SIG, on the other hand, did not seem to affect the levels of estradiol in female rats. Control female rats were found to contain significantly higher levels of LH than those belonging to CYP1, CYP2, SIG1 and MIX2 (p=0.02, 0.0027, 0.031 and 0.0019 respectively). In addition, female of SIG2 group were having higher levels of LH than MIX2 group.

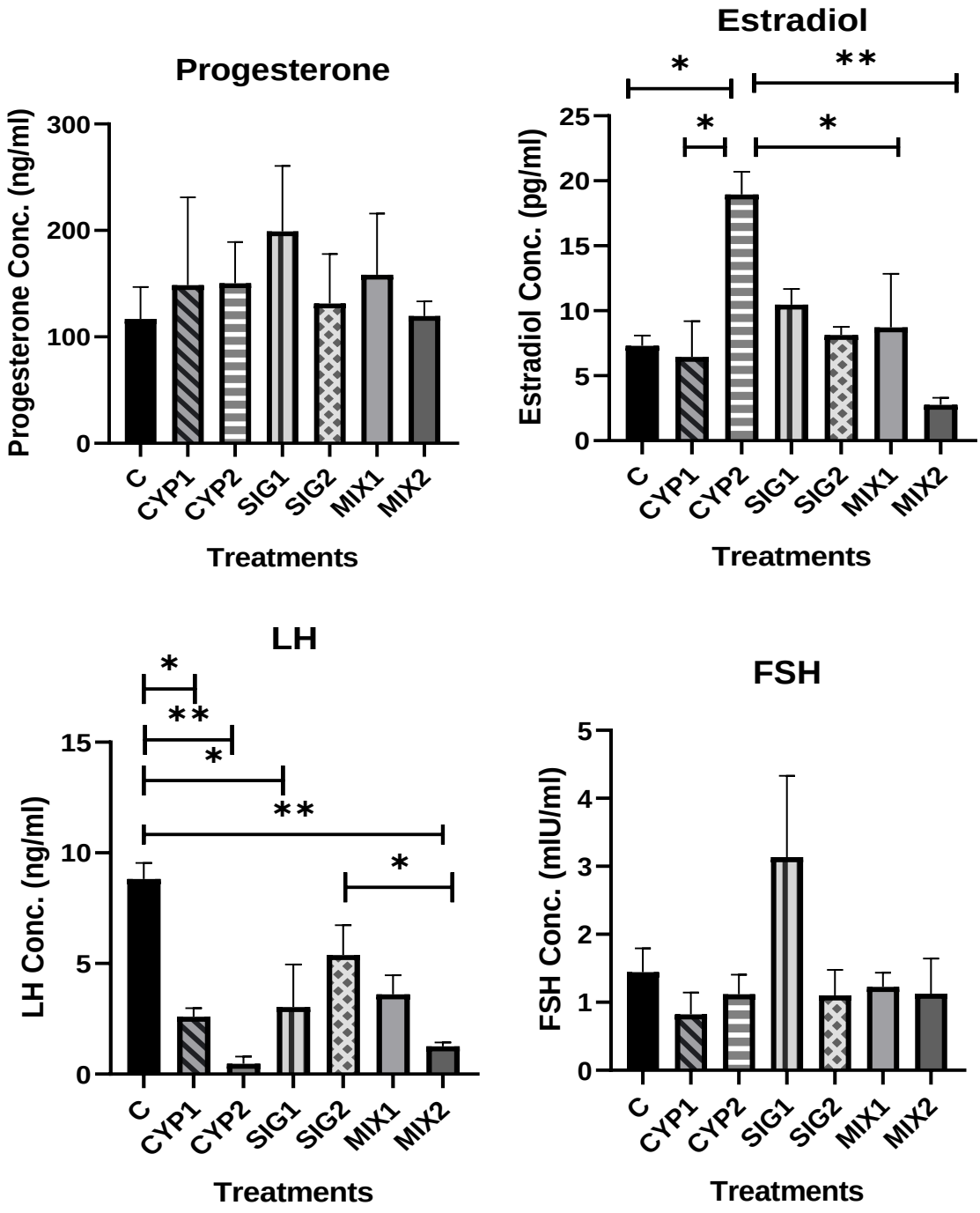

Figure 11: Levels of hormones in female rats subjected to pesticides for 42 days compared to the control. (*p <0.05; ** p <0.01)

Table 5.A shows levels of four hormones (progesterone, estradiol, LH & FSH) in blood samples collected from female offspring rats subjected to pesticides through their mothers during pregnancy and lactation until weaning. LH levels did not show any significant difference between all treatments while, levels of progesterone, estradiol and FSH showed statistical differences between the control and some treatments and between the treatments themselves **(Fig. 12)**. There was no significant difference between the control group and all other groups (except CYP2) with regard to progesterone levels. Progesterone level in CYP2 group was significantly higher than the control. This indicates that the high dose of CYP (20mg/kg bw) significantly increased the level of progesterone in female pups subjected to pesticides via their mothers. In addition, CYP2 group contained higher levels of progesterone than MIX1 and MIX2. Additional differences between groups were also observed; levels of progesterone in MIX2 were significantly less than MIX1, SIG1, SIG2, CYP1 and CYP2. Estradiol levels in all groups (except CYP1) were not affected by the pesticides. CYP1 group showed significantly higher levels of estradiol than C, CYP2 MIX1 and MIX2. FSH levels in all groups (except CYP1) were significantly less than the C group ($P<0.0001$). CYP1 group was found to have significantly higher FSH levels than CYP2 and MIX1.

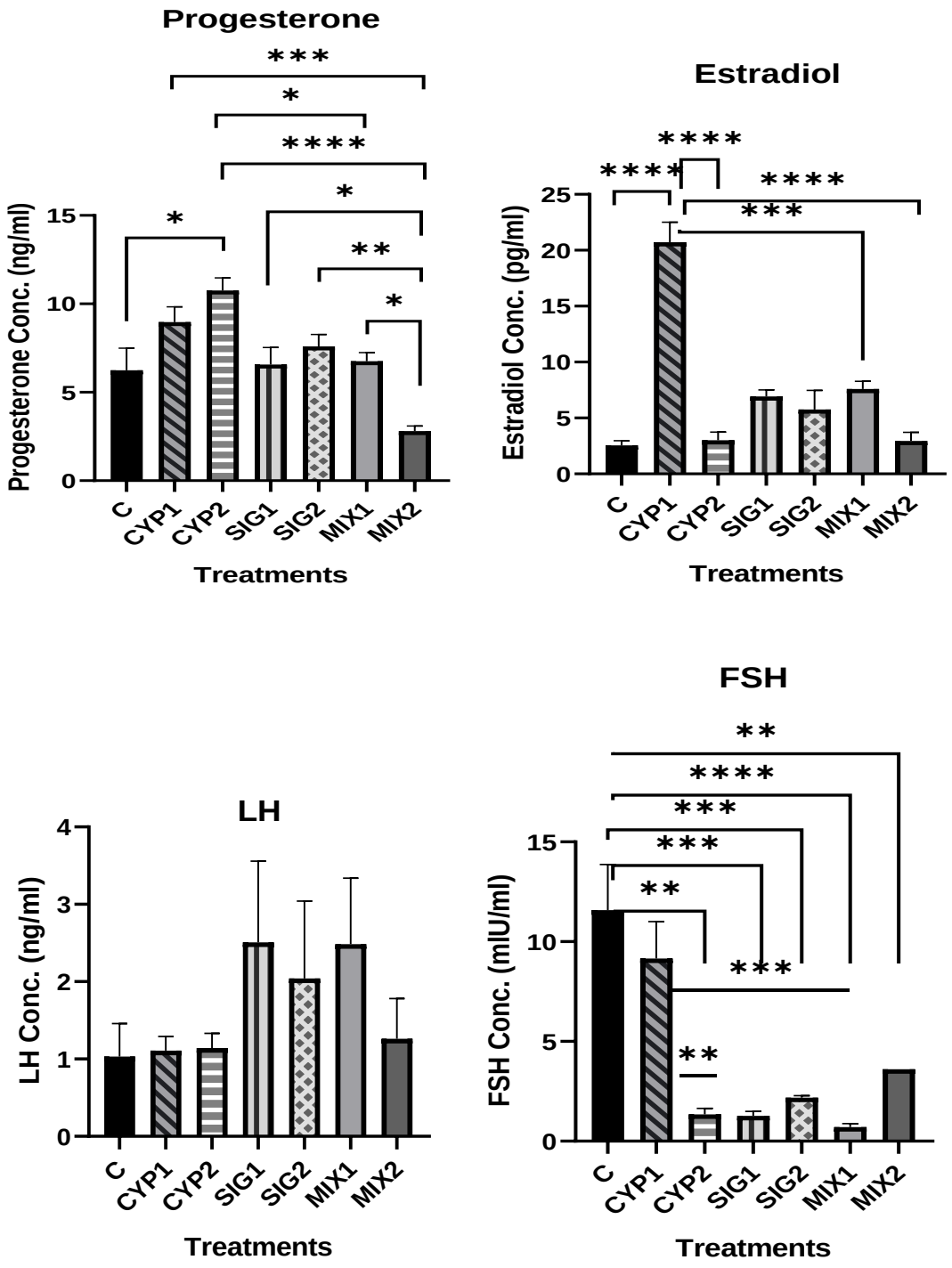

Figure 12: The effect of maternal exposure on the level of pups' female reproductive hormones (Progesterone, Estradiol, LH and FSH). Significant difference: *p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001.

Liu *et al.* (2006) studied the effects of cypermethrin and methyl parathion mixtures on hormone levels in Wistar rats. They reported that the level of estrogen increased from (8.35±7.34 pg/ml) in the control group to (14.16±5.06 pg/ml) when exposed to a mix of pesticides (1.8mg/kg bw CYP and 0.0518 mg/kg bw Methyl Parathion) and to (13.70±4.16 pg/ml) when exposed to (8.00mg/kg bw CYP and 0.23mg/kg bw Methyl Parathion). Estrogen level increased with increased concentration of beta Cypermethrin, from 49.07± 4.93 pg/ml to 55.32 ± 4.03 pg/ml in 5 mg/kg bw group, 66.01 ± 4.43 pg/ml in 10 mg/kg bw and 80.50 ± 7.59 pg/ml in 20 mg/kg bw group (Zhou *et al.* 2018a). In the present study, estradiol was significantly increased in CYP2 group (19.0 pg/ml) compared to the control group (7.3 pg/ml). In female pups, estradiol level was significantly increased in CYP1 group (20.7 pg/ml) compared to the control (2.6 pg/ml). This indicates that cypermethrin causes an increase in estradiol levels whereas, signum did not show such an effect in both adult females and their female pups.

Zhou *et al.* (2018a) studied the effect of cypermethrin exposure on the reproductive function of female mice. They reported that the level of progesterone decreased from (10.94±1.32 pg/ml) in control group to 9.34±0.66 pg/ml in the group subjected to 5mg/kg bw and to 8.19±0.50 pg/ml in the group exposed to 10mg/kg bw and to 6.83±0.83 pg/ml in 20mg/kg bw exposed group. On the other hand, Obinna and Kagbo (2017) and Obinna and Agu (2019) did not find any effect of cypermethrin on progesterone in female albino rats. Their findings are in agreement with results of the present study.

Some studies reported that after exposure to CYP, levels of FSH decreased while others reported an increase. Liu *et al.* (2006) found that levels of FSH in female Wistar rats decreased after combined exposure to cypermethrin and methyl parathion. These findings are in agreement with our results obtained from female pups where a significant decrease in FSH level was observed

upon exposure to pesticides. On the other hand, FSH level was found to increase upon exposure of mice to cypermethrin (Zhou *et al.* 2018a).

Zhou *et al.* (2018a) reported that levels of LH significantly decreased upon exposure to cypermethrin. They found that the level of LH decreased from 3.54±0.24 mlU/ml in control group to 2.19±0.37 mlU/ml in the group exposed to 2.76 mg/kg bw CYP and to 1.18±0.17 mlU/ml in the group exposed to 5.52 mg/kg bw CYP. These results fully agree what our results obtained from adult female rats exposed to CYP as our results show that both CYP and SIG and MIX2 significantly decrease the levels of maternal LH. No such effect was observed in female pups exposed to any treatment of pesticide. These results indicates that both CYP and SIG interfere with female reproductive hormones (LH and estradiol).

For parent serum hormonal analysis, both estrogen and LH level were altered. High dose of Cypermethrin CYP2 shows an increase in estradiol level in parents' serum. estrogen is responsible for implantation and readying the uterine for implantation, a small amount of estrogen is enough, it is even better to have small estrogen concentration than a very high one this is because studies reported that low estrogen concentration extend the time of implantation and uterine receptivity while a high dose rapidly close that opportunity thus decreasing the implantation rate which was seen in literature (Zhou *et al*. 2018b). By looking to our data the groups treated with Signum and a low dose mixture had normal estrogen level and they were the groups which few females accidentally got pregnant for the second time. The LH level in parents serum decreased in groups treated with Cypermethrin, low dose of Signum and the high dose of the mixture compared to the control. low level of LH effect the maturation and the development of follicles (Zhou *et al.* 2018a) which is seen in the case of high dose of Cypermethrin where there was a decrease in the number of follicles compared to the control groups **Figure (22),** as for the groups treated with Signum

there was a significant decrease in LH level in the low dose of sig while there was no effect on group treated with the high dose of sig this might be due to experimental error.

In pups serum, the levels of both progesterone and estrogen are elevated in groups treated with Cypermethrin. As mentioned above, the high level of estrogen decrease the chance of implantation (Zhou *et al.* 2018). The level of FSH was reduced in all treated groups, low levels of FSH can be due to cystic ovaries, incomplete puberty, and gonadotropin deficiency (You and Your Hormones, Society for Endocrinology (2018)). An elevated progesterone levels leads to stop eggs developments and stops the development of follicles (Komatsu, 2017).

As for the differences of affected hormones between mothers and their pups, this might be because the pups are in the developmental stage so pesticides might affect the pituitary gland, this can lead to the changes seen in our experiment. The timing of the exposure can be a cause of the differences between hormones level in mothers and pups. The exposure to endocrine disruptor during early stages of development can be extremely dangerous and causes a more pronounced effects, thus a maternal exposure led to a significant changes to pups (Sweeney, *et al* 2015, Lauretta. *et al* 2019).

Generally, it can be concluded that both pesticides and their combinations can affect the reproductive hormones in either adult female rats or female pups or in both.

4.3 Effect of CYP and SIG on ovaries morphology:

Effects on ovaries of mother rats:

Figure (13) shows the ovarian histology of mother rats at the end of the experiment. Normal ovarian tissues of the control group can be seen in **Fig. 13A**. It shows healthy- looking ovarian tissue with well-developed follicles. Ovaries of female rat groups exposed to CYP2 and SIG2 (**Fig. 13 B & C**, respectively) appear to have lower follicular development and unhealthy-looking tissues compared to the control group. As for the MIX 2 group (**Fig. 13D**), the ovary size was very small, almost half the size of other groups.

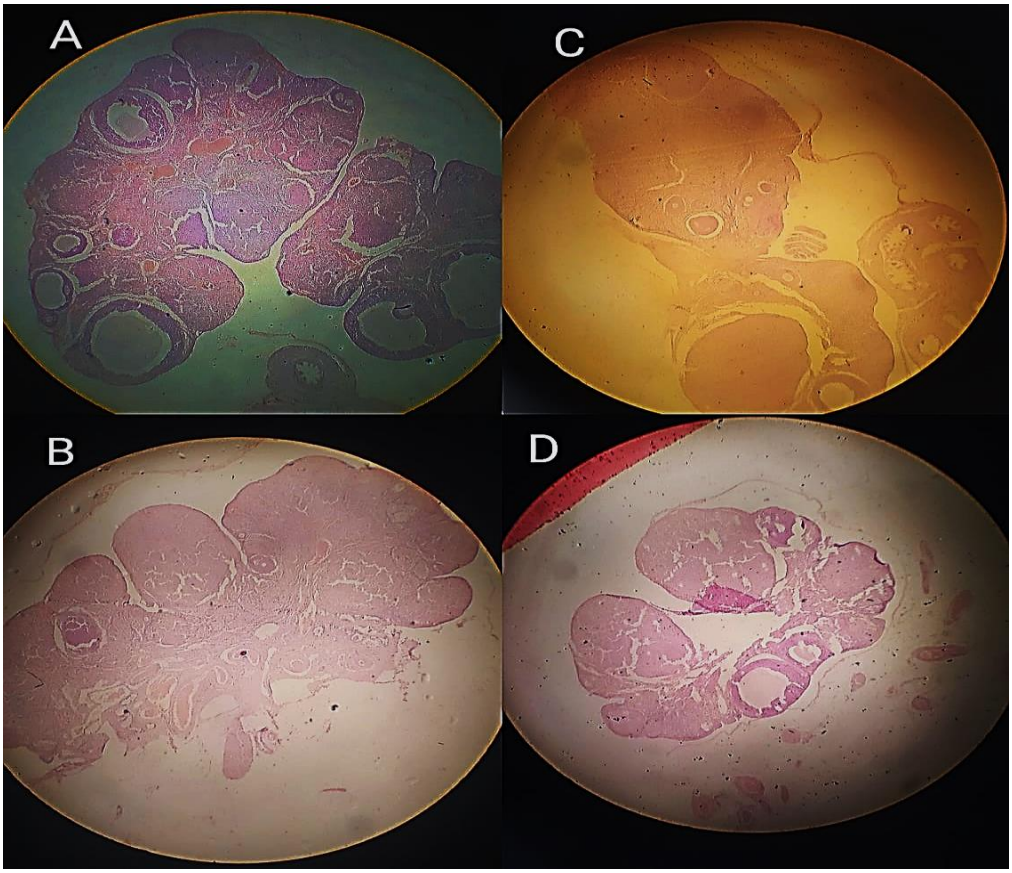

Figure 13: Ovarian histology of mother rats (4X). (A) Control group, (B) CYP2 exposed group, (C) SIG2 exposed group and (D) MIX2 exposed group.

Figure (14) shows different ovarian histological sections of the control mother rats. Sections show different stages of follicles (primary follicles (PF), secondary follicles (SF) and tertiary follicles (TF)) in addition to corpus luteum (CL), normal blood vessels (BV), normal granulosa cells and oocytes. **Figure (15)** shows different ovarian sections of a mother rat from group CYP2. It appears to have less secondary and tertiary follicles compared to the control group. Besides, it demonstrates the presence of congested blood vessels.

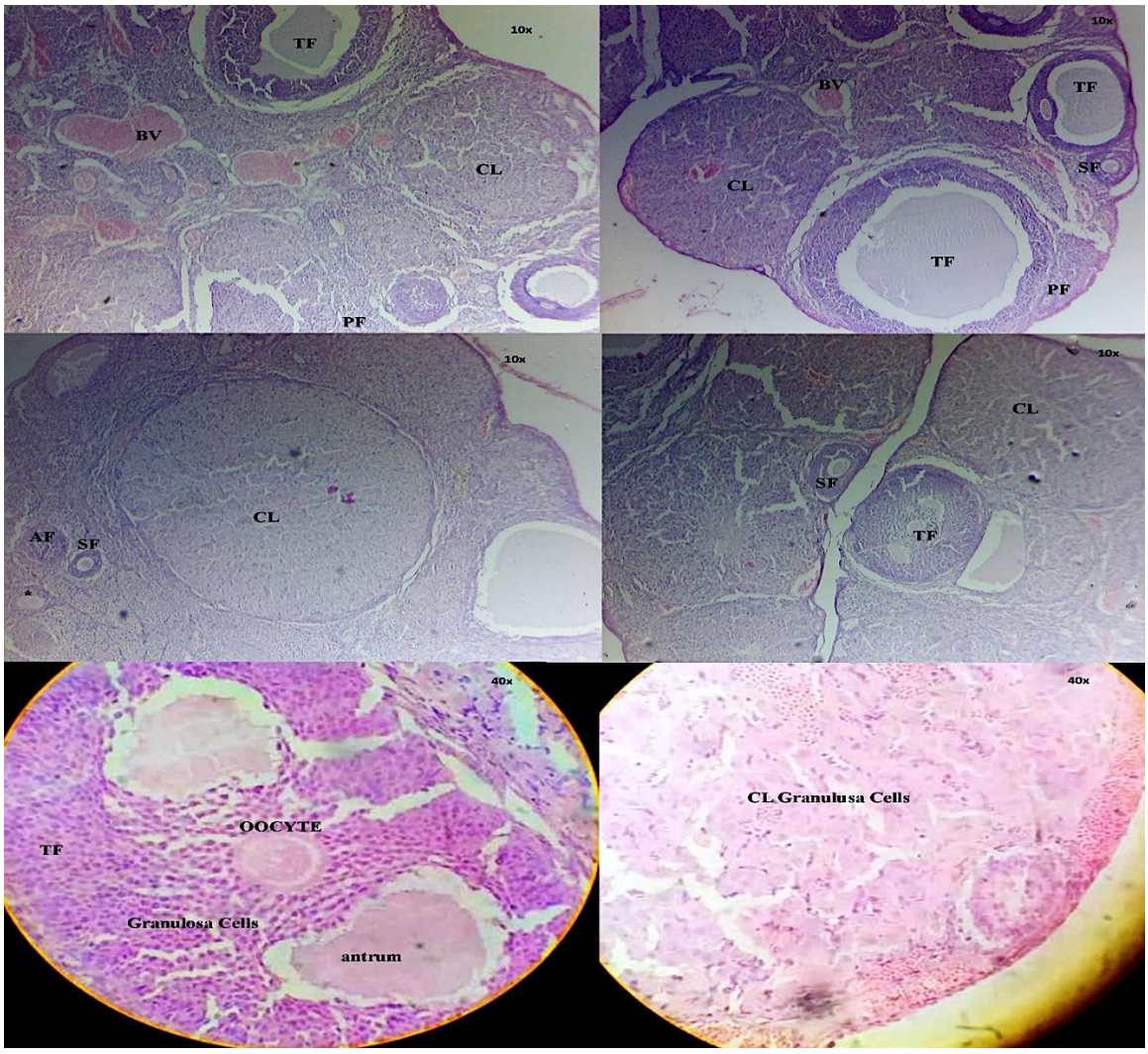

Figure 14: Ovarian histology of control mother rats showing normal blood vessels, granulosa cells, oocyte and follicles development. (Pf: primary follicle, SF: secondary follicle, TF: tertiary follicle, CL: corpus luteum, BV: blood vessels).

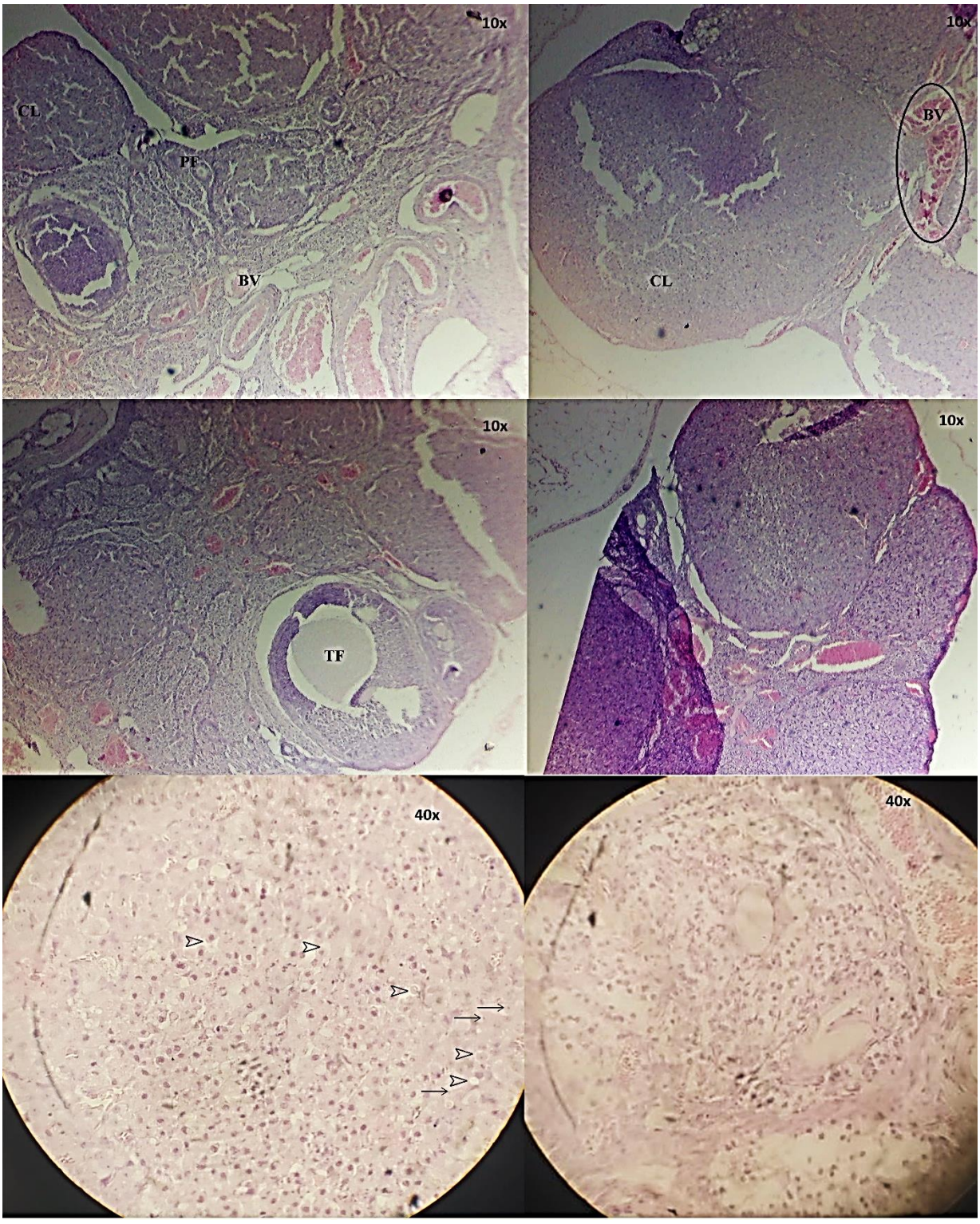

Figure 15: Ovarian histology of CYP2 mother rats showing congested blood vessels, micronuclei (arrows) and vacuolated cells (arrows heads). TF: tertiary follicle, CL: corpus luteum, BV: blood vessels.

The ovaries of mothers from groups exposed to CYP2, SIG2 and MIX2 show the development of micronuclei and vacuolated cells **Figure (15, 16 & 17)**. The ovaries of SIG2 group also indicated the presence of atretic follicle **Figure (17)**.

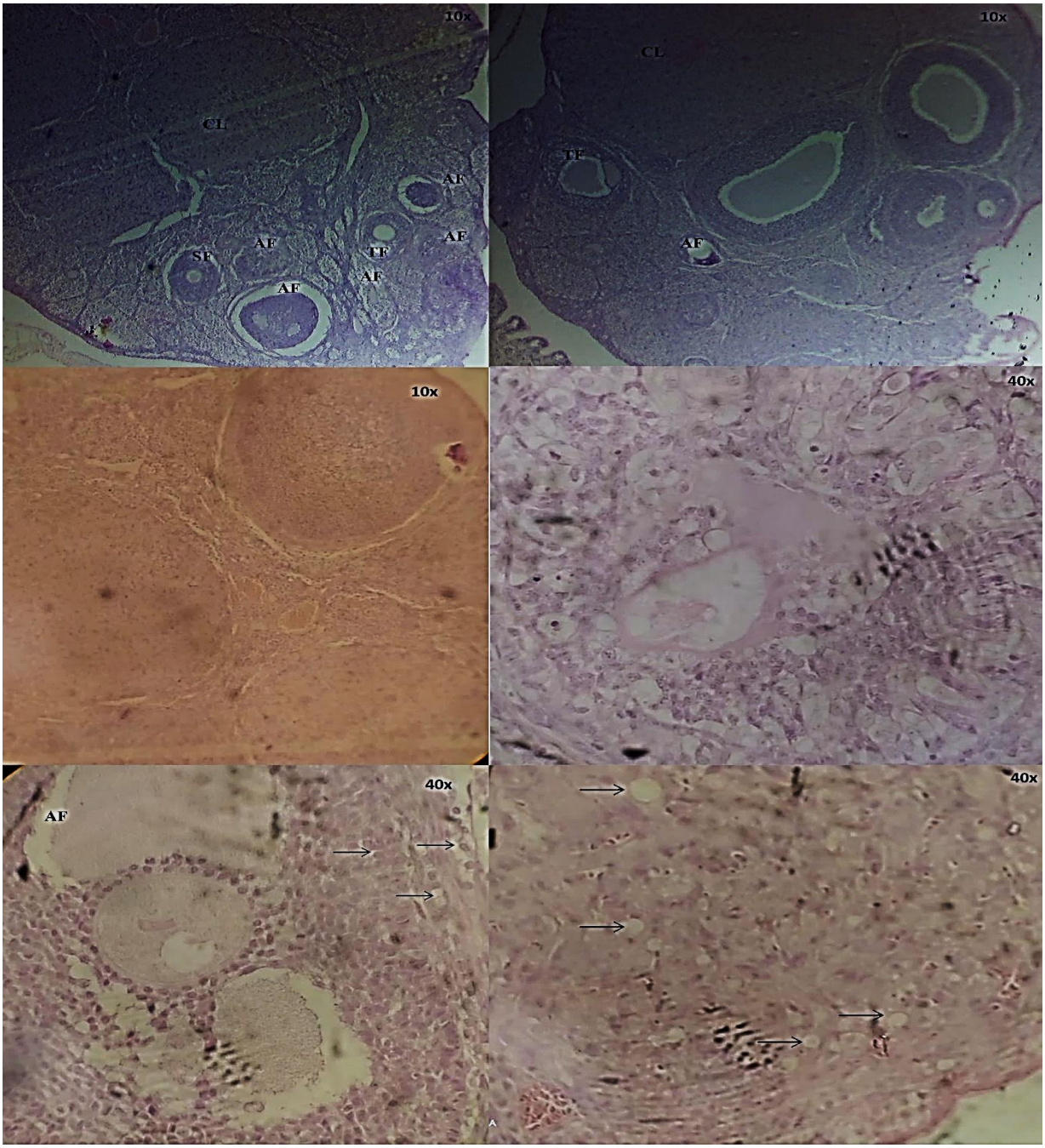

Figure 16: Ovarian histology of SIG2 mother rats showing vacuolated granulosa cells (arrows). (SF: secondary follicle, TF: tertiary follicle, CL: corpus luteum, BV: blood vessels).

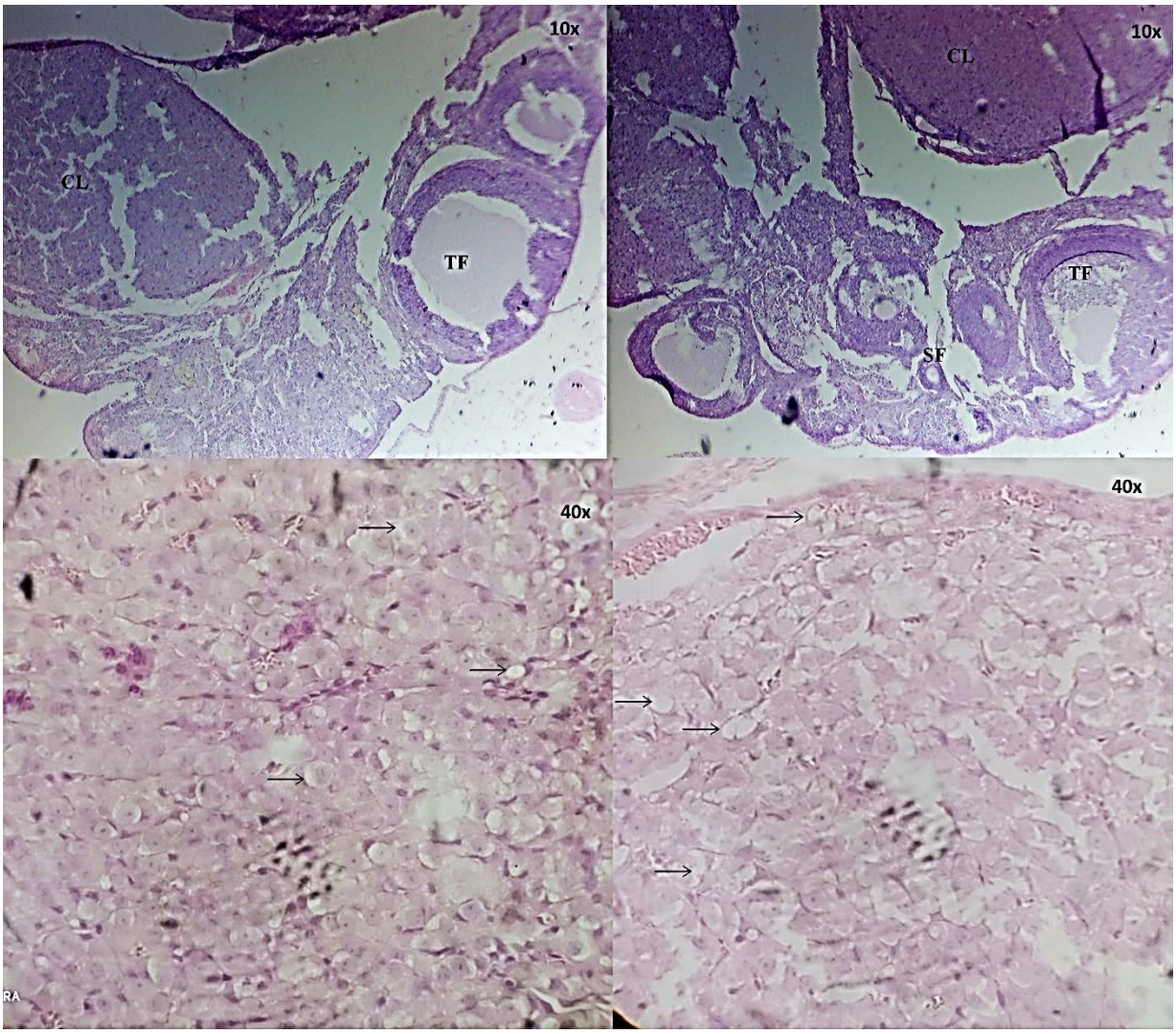

Figure 17: Ovarian histology of MIX2 mother rats showing vacuolated granulosa cells (arrows). (SF: secondary follicle, TF: tertiary follicle, CL: corpus luteum).

Effects on ovaries of pups' female rats:

Figure (18) shows ovarian histology of the control female pups. The figure shows different stages of follicles (PF, SF & TF), corpus luteum, normal blood vessels, normal granulosa cells and oocytes. **Figure (19)** shows ovarian histology of pups from CYP2 group where congested blood vessels, micronuclei and vacuolated cells are observed. Even though the ovaries of pups from SIG2

and MIX2 show normal developed follicles; vacuolated cells as well as atretic follicles were seen too (**Figures 20 & 21).**

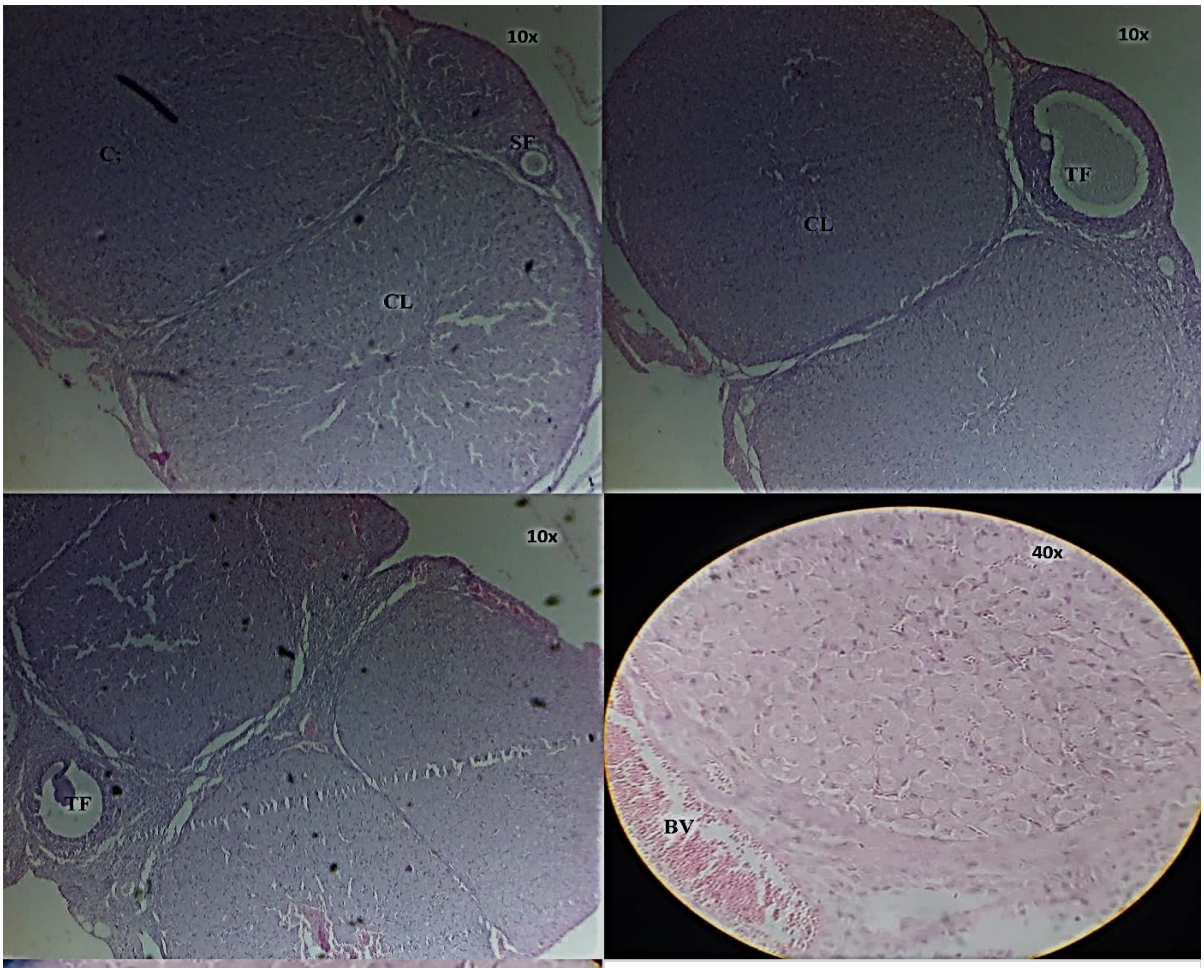

Figure 18: Ovarian histology for pups of the control group showing normal granulosa cells with normally developed follicles. (SF: secondary follicle, TF: tertiary follicle, CL: corpus luteum, BV: blood vessels).

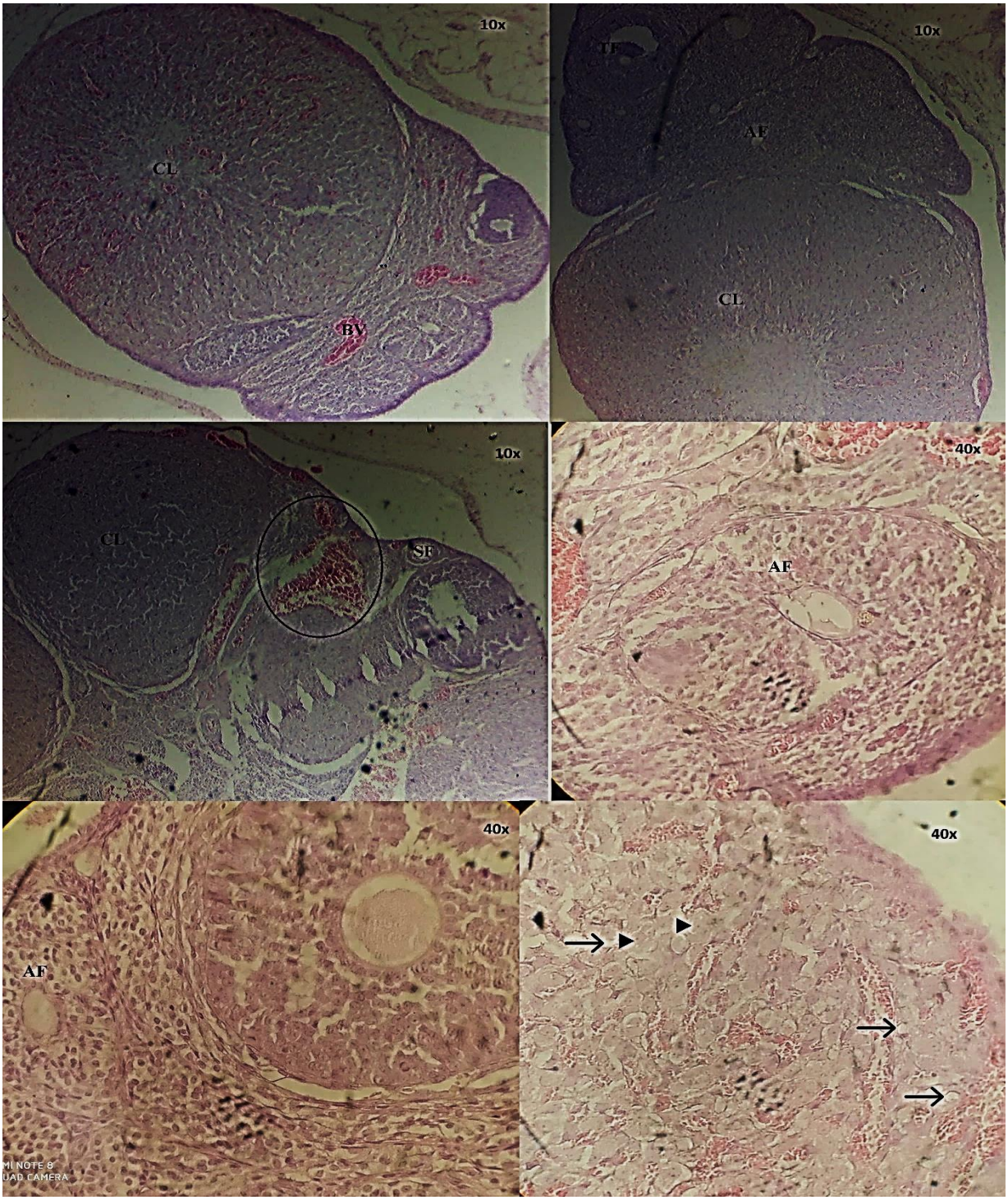

Figure 19: Ovarian histology CYP2 pups group showing congested blood vessels, vacuolated granulosa cells (arrows) micronuclei (arrows heads) and atretic follicles (AF).

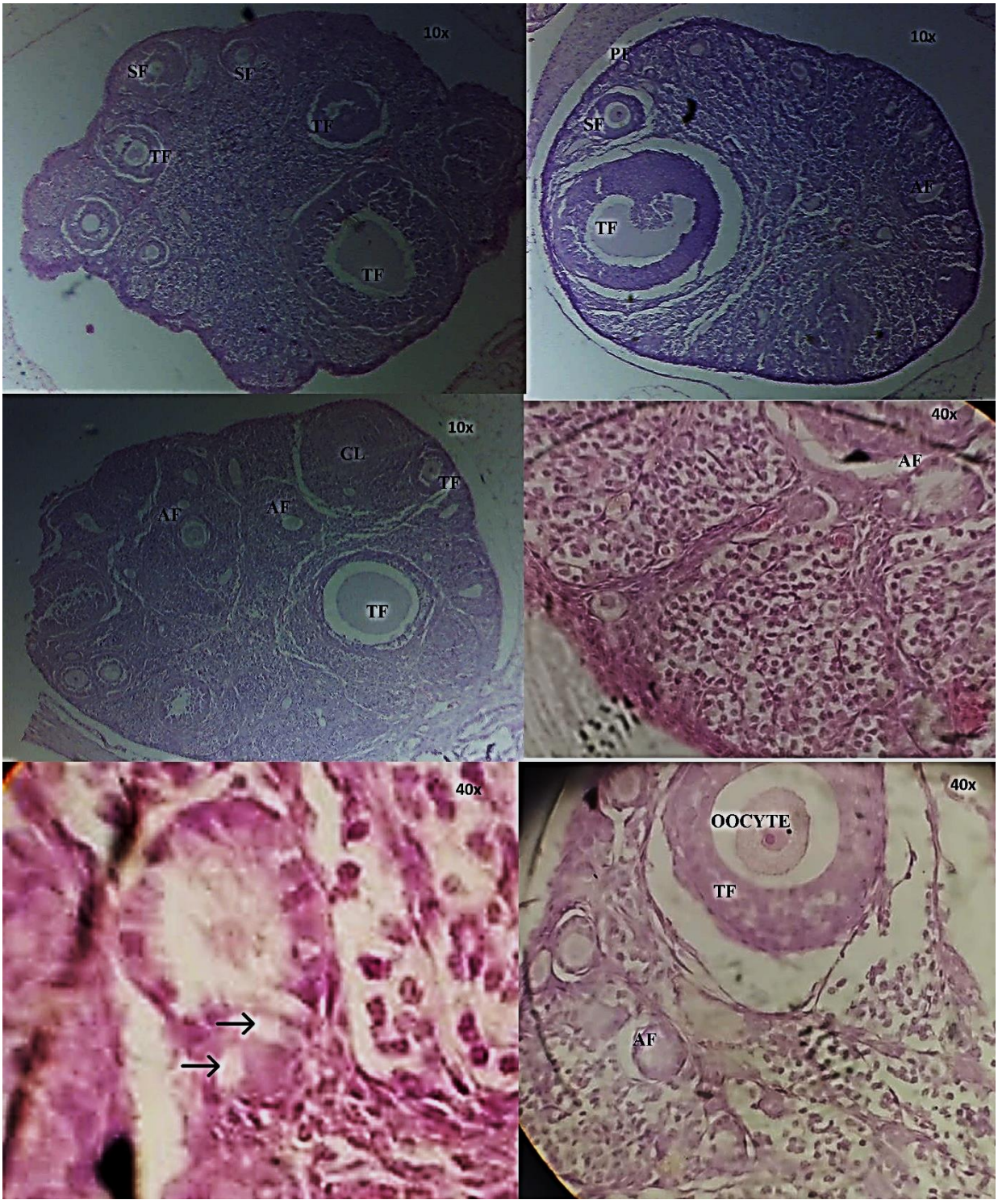

Figure 20: Ovarian histology for pups of SIG2 group showing vacuolated cells (arrows), atretic follicles (AF). (Pf: primary follicle, SF: secondary follicle, TF: tertiary follicle, CL: corpus luteum).

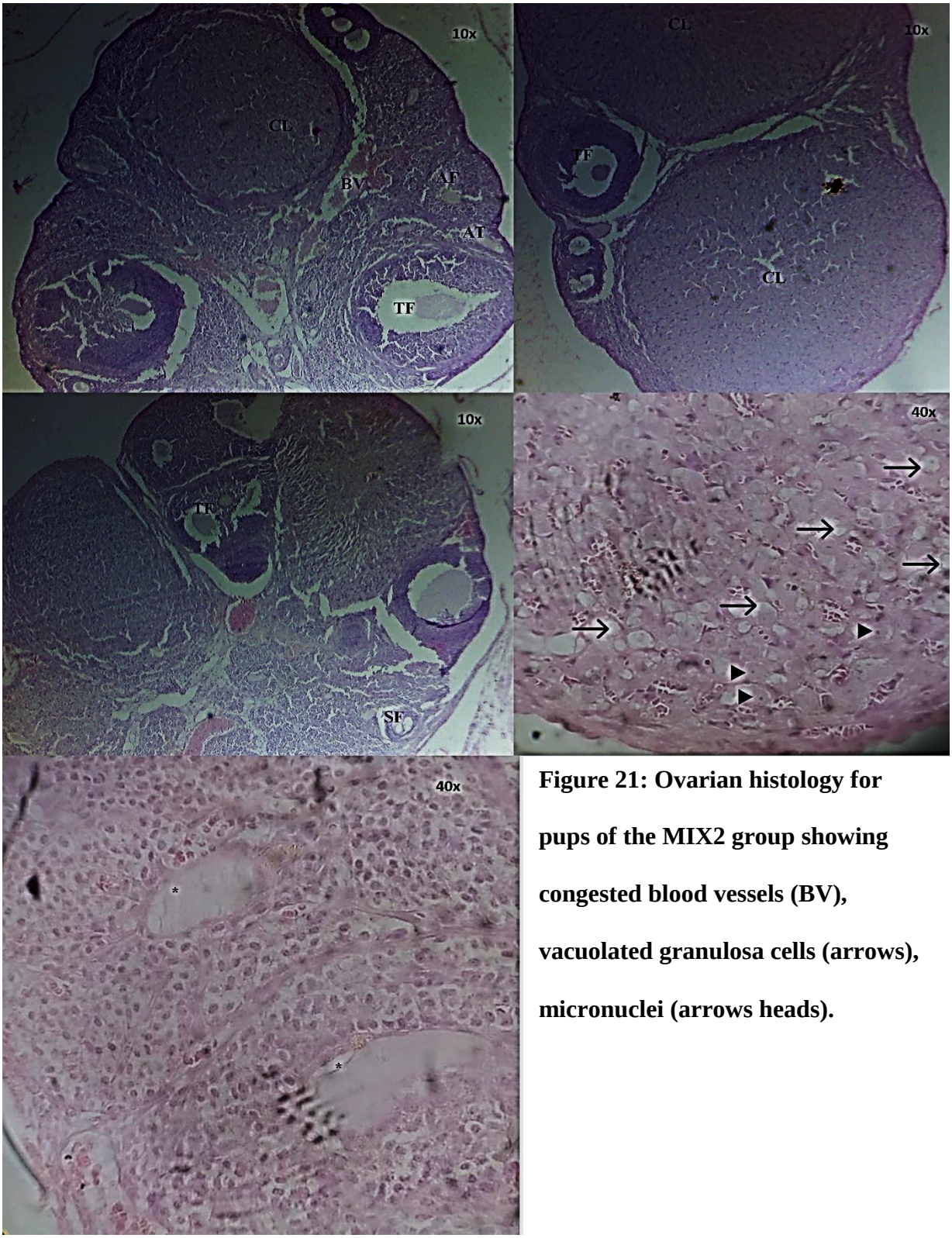

Figure 21: Ovarian histology for pups of the MIX2 group showing congested blood vessels (BV), vacuolated granulosa cells (arrows), micronuclei (arrows heads).

Histopathological examination of the mothers' and pups' ovarian tissues indicated a statistically significant decrease in the total number of normal follicles especially in CYP2 groups compared to the control (P=0.02, 0.0318, respectively) (**Table 6. A & Figure 22**) where the number decreased from 25.7 in the control mothers' group to 9.3 in the CYP2 treated mothers' group. SIG2 and MIX2 were having a total number of normal follicles that is 12.0 and 13, respectively. In the pups' group, the mean number of normal follicles decreased from 21.5 to 9.0. While SIG2 and MIX2 were having 20.7 and 16.0, respectively. These results indicate a significant effect of CYP on total number of normal follicles in both mothers and pups.

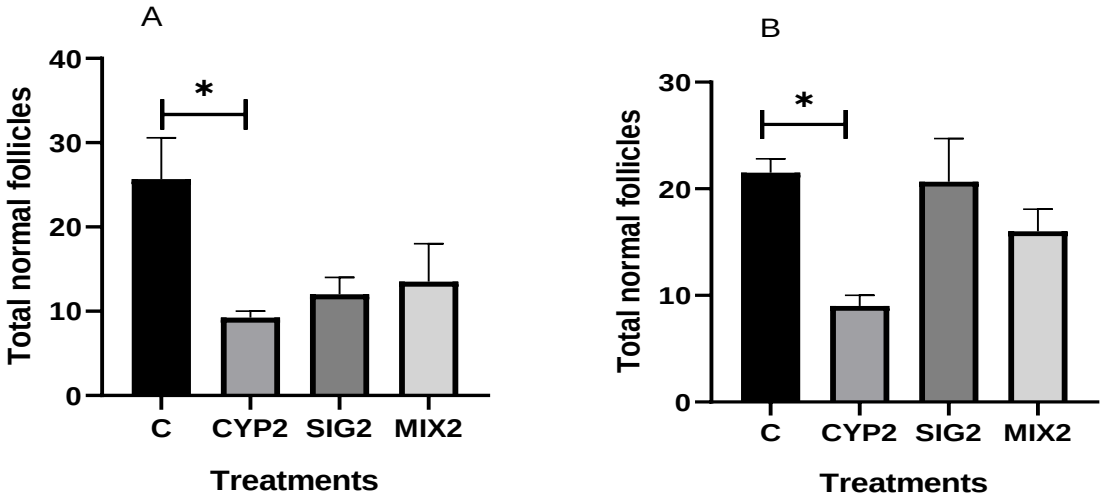

Figure 22: Total number (MEAN±SEM) of normal follicles in female rats belonging to the four groups of treatments. (A) Mothers, (B) Pups. (*) indicate a significant difference ($p<0.05$).

Table 7.A summarizes the numbers of primary follicles, secondary follicles, Graafian follicles and corpus luteum in mothers' ovarian histological sections at the end of the experiment. Statistical analysis showed that there was no significant difference neither in the number of primary follicles nor in the corpus luteum between all four groups **(Fig. 23**). Results indicated a significant decrease in the number of secondary follicles in CYP2 group compared to the control (P=0.0013) and SIG2

groups (p=0.028). The number decreased from 3.8 and 3.0 in the control and SIG2, respectively, to only 1 in CYP2 group. The Mean number of Graafian follicles in CYP2 (0.5) was statistical less than that of the control group (3.5) (P=0.0143). Although less than the control, other groups did not show any statistically significant difference in the number of Graafian follicles.

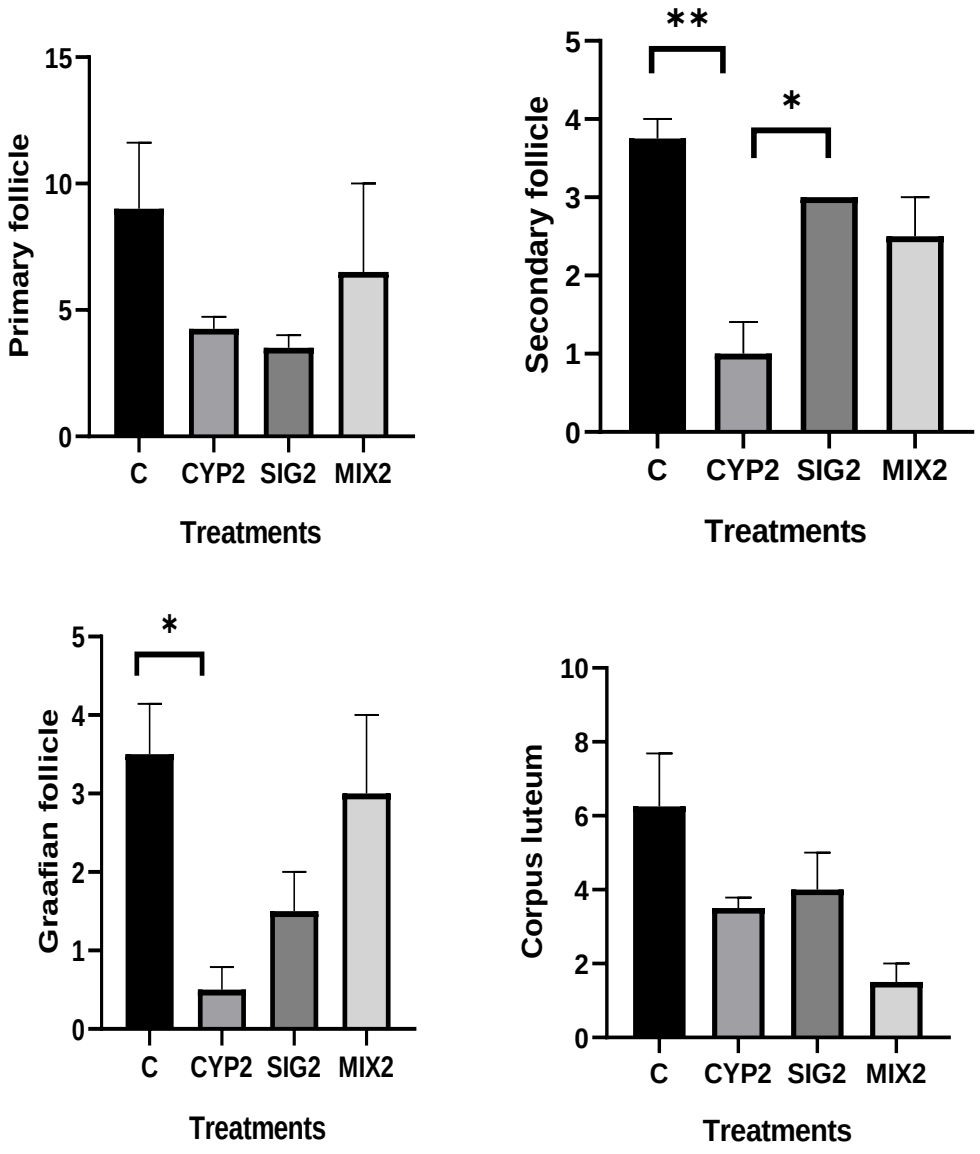

Figure 23: Numbers (MEAN±SEM) of primary follicles, secondary follicles, Graafian follicles and corpus luteum in mothers' ovarian histological sections at the end of the experiment. Significant difference: *p <0.05, ** p <0.01.

Table 8.A summarizes the numbers of primary follicles, secondary follicles, Graafian follicles and corpus luteum in pups' ovarian histological sections at the end of the experiment (about 1 month of age). Results indicated no significant difference in the number of primary or secondary follicle between the four groups (**Fig. 24).** The number of Graafian follicles was significantly reduced in CYP2 group (1.0) compared to the control (3.5) and MIX2 (3.5) (P=0.018). In addition, the number of corpus luteum in SIG2 (0.5) was statistically less than that of the control (4.8) (P=0.0049).

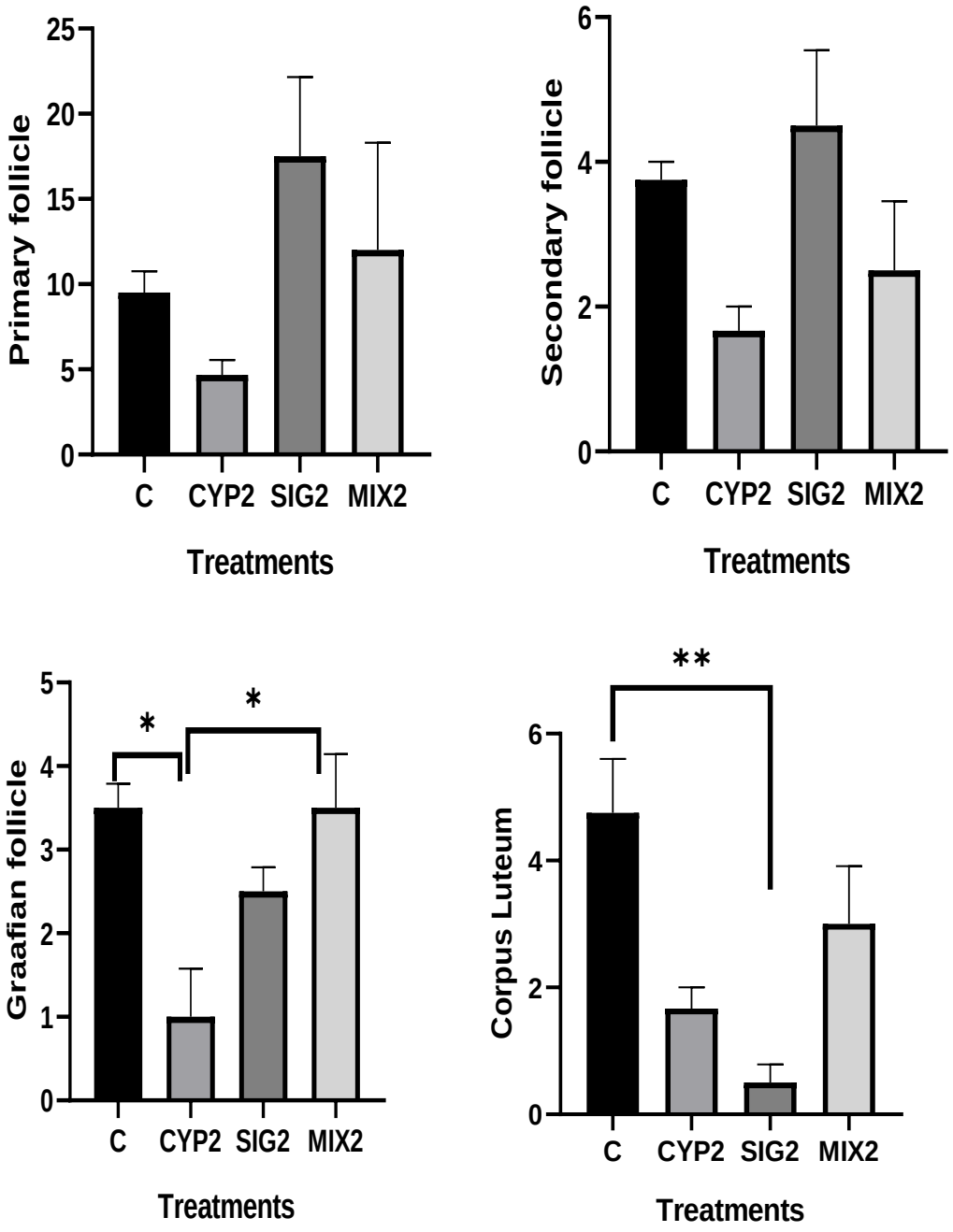

Figure 24: Number (MEAN±SEM) of primary, secondary and Graafian follicles in addition to corpus luteum in ovaries of pups' groups of four treatments (1 month old). Significant difference: *p <0.05, ** p <0.01.

The percentage of the atretic follicles in both mothers and pups' groups are summarized in (**Table 9.A)**. A significant increase in the percentage of atretic follicles in SIG2 mothers' group (51.1%)

was observed in comparison with the control (15.5%) and MIX2 (19.8%) (P=0.0014 and 0.0049 respectively). The pups' groups showed an increase in the percentage of atretic follicles compared to the control, however, this increase was not statistically significant (**Fig. 25).**

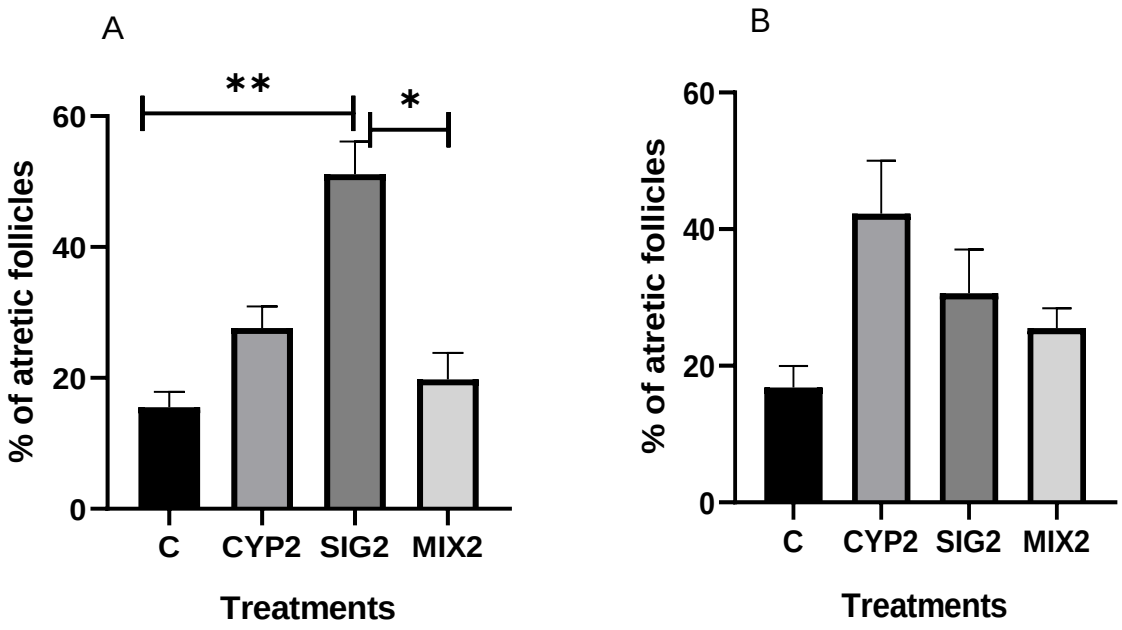

Figure 25: The percentage of atretic follicles. (A) Mothers atretic follicles. (B) Pups atretic follicles. Significant difference: *p <0.05, ** p <0.01.

In an experiment on female mice subjected to different doses of CYP, Al-Hamadani and Yajurvedi (2017) reported a decrease in ovarian size, decrease in the total number of healthy follicles, a significant reduction in the number of estrous cycles per month, serum levels of estradiol, total number of healthy ovarian follicles and number of corpora lutea. The number of atretic follicles was found to increase in all groups compared to control group. Some of these results are in agreement with our results represented by **Figs. 22-24.** Zhou *et al.* (2018a) and Wang *et al.* (2019) also reported a decrease in the number of follicles. An increase in the number of atretic follicles in groups exposed to CYP was also reported by Sangha *et al.* (2013) and Singh *et al.* (2019). Other

studies reported that the CYP exposure causes a reduction in primary follicles as well as secondary and corpora lutea (Singh *et al.*, 2019).

Studies on the impact of SIG on reproductive biology of living organisms are rare. Cayir *et al.* (2014) reported from an invitro study that Signum causes the development of micronuclei in human lymphocytes. This finding is in agreement with ours that SIG causes the development of micronuclei in the granulosa cells of the corpora lutea. Another study on zebra-fish reported that there was a 16% decrease in late oocytes in the ovaries of zebra fish due to the exposure to 1.0 mg/L boscalid (Qian *et al.*, 2020).

Based on the results of the present study, both pesticides affect the ovaries negatively and can cause decreased follicles number, vacuolated cells and/or micronuclei cells as well as decrease in the size of the ovaries. As for the groups treated with high dose mixture, the histologically examination was hard because the specimen was small and was disfigured so this might be the cause of what looked like normal follicles number.

4.4 Genotoxic effect of CYP and SIG:

The consistency of RAPD method was checked (**Fig. 26**) and the results confirmed that the isolated DNA gives always the same banding pattern with the same primers; which indicates good integrity of the DNA isolated. **Figure 27** shows some of the banding patterns obtained by the primers used. 12, out of 13 primers used, generated polymorphic bands (**Table 5**). Total polymorphic bands generated was 73 with an average of 6 bands/primer (range 1-16 bands/primer). Polymorphic bands represented 25% of all bands generated after exposure to pesticides. The total number of bands that disappeared was 44, while that of the newly appearing bands was 29 **(Table 6)**. These results indicate that both pesticides are mutagenic. However, CYP (average 7 polymorphic bands/2 doses) seems to be less mutagenic than SIG (average about 15 polymorphic bands/2 doses) and the MIX (average 14 bands/2 doses).

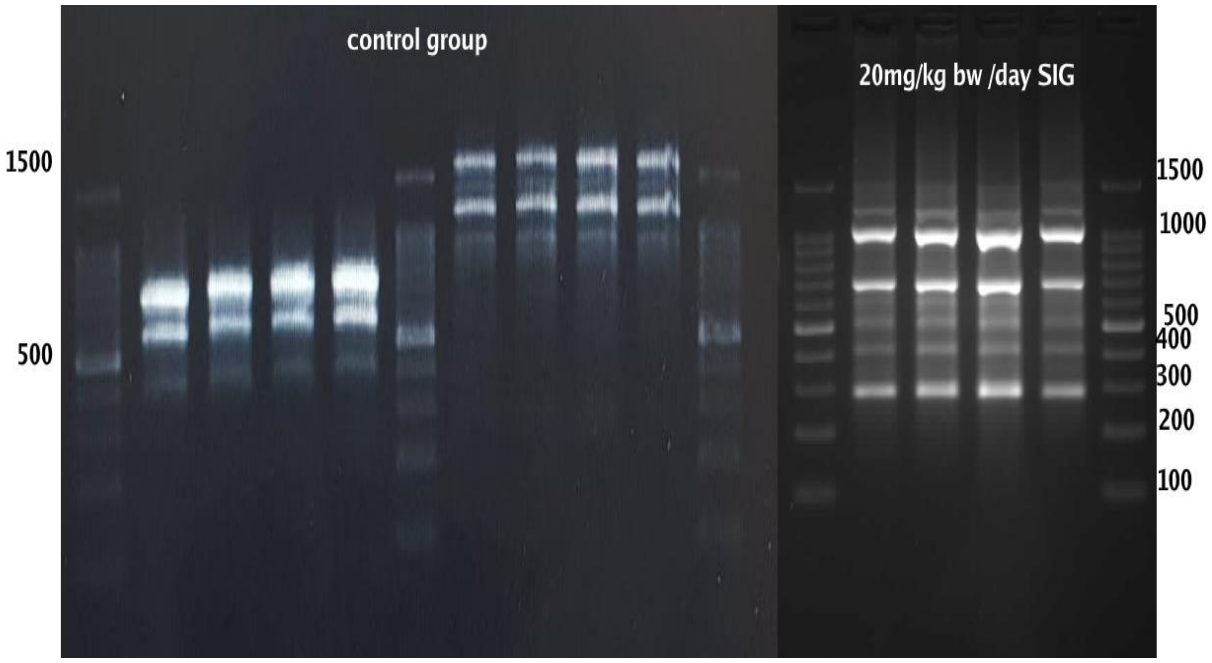

Figure 26: Reproducibility of RAPD profiles

Table (5): Polymorphic bands generated by the primers used in the present study

Primer	Sequence 5`-3`	Total bands		Polymorphic bands
		Before exposure	After exposure	
A04	AAT CGG GCT G	32	25	8
C1	TGC GCC CTT C	16	15	3
C06	GAA CGG ACT C	18	18	0
PG3	GCA TGC GAT C	40	41	16
A03	AGT CAG CCA C	18	15	3
APO8	ATG CAG GCT T	35	36	1
B9	GTT TCG CTC C	23	18	11
PA09	TCT GCT CTC C	18	16	4
PA02	GAC CAT TGC C	34	37	3
04	AGG GCC CGG G	20	19	7
PG5	TTC GAC CCA G	10	13	3
PG9	GCT GCT CGA G	20	18	4
PG12	CCA GCC GAA C	18	16	10
	Total bands	**302**	**287**	**73**

Table (6): The Total number of bands and polymorphic bands generated by RAPD analysis of rat's DNA obtained from each treatment.

Groups	Total bands		Bands		
	Before exposure	After exposure	Appeared	Disappeared	Total polymorphic
C	39	39	0	0	0
CYP1	39	41	4	2	6
CYP2	31	33	5	3	8
SIG1	54	44	6	16	22
SIG2	46	41	2	7	9
MIX1	47	42	4	9	13
MIX2	46	47	8	7	15
Total	**302**	**287**	**29**	**44**	**73**

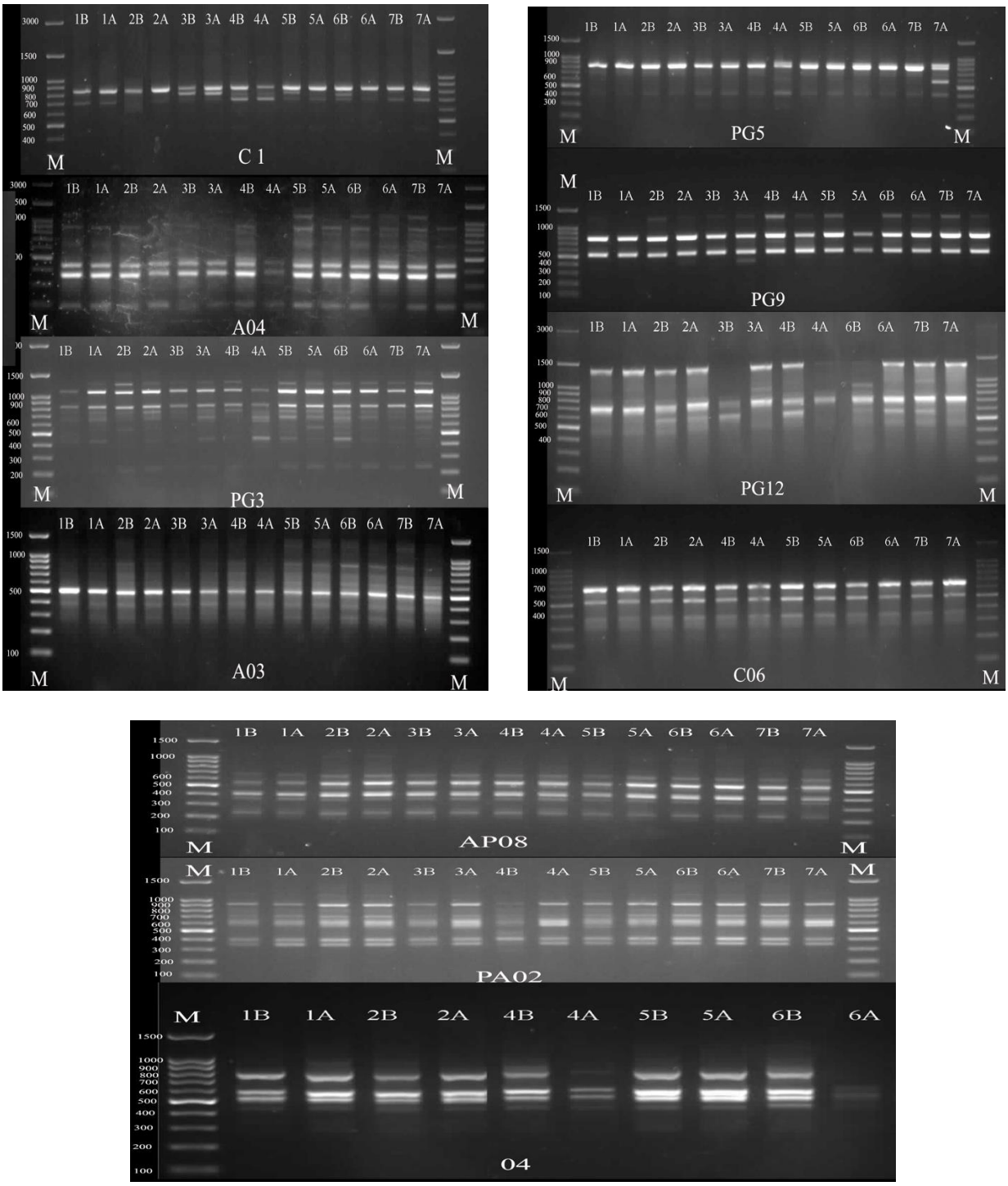

Figure 27: Some RAPD fingerprinting obtained using different primers to evaluate genotoxicity of the pesticides. B: Before exposure, A: After exposure. Numbers represent group numbers 1: Control, 2: CYP1, 3: CYP2, 4: SIG1, 5: SIG2, 6: MIX1, 7: MIX2, M: Marker.

Similarity indices, which measure the proportion of shared fragments of DNA in the amplification profiles, were calculated and are shown in **Fig. 28**. Results indicated similarity indices for the treatments ranging between 94.28 (CYP1) and 77.92 (SIG1). The average similarity indices of both doses together were 90.31, 84.91 and 83.57 for CYP, SIG and MIX, respectively. These results indicate genotoxicity of all treatments and doses of the pesticides and mixing the insecticide and fungicides together increases their genotoxicity.

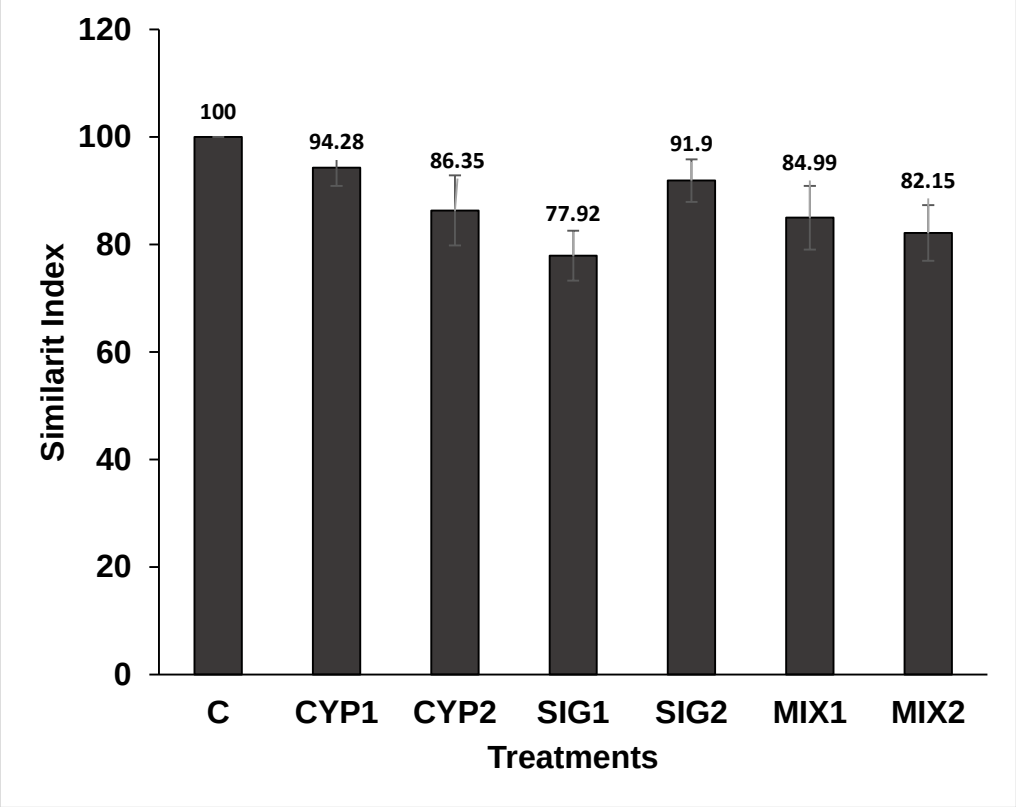

Figure 28: Genetic similarity between rats before and after exposure to pesticides.

According to previous data, both pesticides were genotoxic and genotoxicity seems to be dose-dependent at least in the case of CYP (94.5% and 86.9%) and MIX (86.1% and 83.5%) treated groups. According to Amer *et al*, (1993), there was a dose-dependent increase in the frequency of sister chromatids exchange when bone marrow cells were exposed to 300 mg/kg cypermethrin (11.12 ± 0.05) compared to solvent and control groups (3.7 ± 0.14 and 4.4 ± 0.26) respectively. In mice spleen cells exposed to 4.00 µg/ml cypermethrin, sister chromatids exchange reached 15.1 ± 0.05 compared to solvent and control groups (8.6 ± 0.23 and 5.9 ± 0.39, respectively (Patel *et al.*, 2006). They also reported that there was a dose-dependent increase in DNA damage in several organs of mice (spleen, kidney, liver, bone marrow, lymphocytes and brain) subjected to cypermethrin. Kocaman & Topaktaş (2009), also confirmed the genotoxicity of cypermethrin where they reported that the sister chromatid exchange and chromosomal aberrations were significantly induced in lymphocyte cells treated with 5, 10, 15 and 20 µg/ml cypermethrin for 24 and 48 h. They also reported the significant induction of micronuclei at 5 and 10 µg/ml of cypermethrin. Cayir, *et al.* (2014) reported that micronuclei formation in human lymphocytes increased significantly at doses 2, 6, 25 µg/ml Signum. In addition, the nucleoplasmic bridges significantly increased at a dose of 0.25 µg/ml pyraclostrobin. According to Zhang *et al.* (2017), pyraclostrobin was found to be genotoxic to zebra-fish. They reported that there was a significant dose-dependent increase in DNA damage in exposed fish. These findings are in agreement with our finding of the genotoxicity of both tested pesticides.

4.5 CYP & SIG residues in blood samples of F1 offspring:

Residues of both pesticides in blood samples of offspring were not detectable. These results confirm that a major fraction of the two pesticides and their metabolites are quickly excreted from blood and another smaller one remains stored in fat tissues (Australian Pesticides and Veterinary Medicines Authority 2004). Some studies also reported that Cypermethrin residues can be found in skin, hair and digestive content (Crawford et al. 1981; K. Kim et al. 2008; Rhodes et al. 1984). According to different studies pyraclostrobin is converted to other metabolites i.e. N-desmethoxy metabolite and its residues were mostly found in liver, fat and milk of lactating goat after 23Hrs of exposure (Australian Pesticides and Veterinary Medicines Authority 2003). As for boscalid, its residues can be also found in fat and milk of lactating goat as well as in fat of eggs of hens (Australian Pesticides and Veterinary Medicines Authority 2004).

Studies on rats show that 50% of ingested pyraclostrobin is absorbed by the body with peaks appears in the plasma in the first 30 minutes then 8 or 24 hours after exposure. After 2 days (74-91%) of ingested pyraclostrobin was excreted via faeces and (10-13%) via urine. In vitro studies, rats and humans skin were exposed to different concentration of pyraclostrobin; (21-51%) and (3-8%) of the doses were absorbed respectively after 24hour of exposure (Australian Pesticides and Veterinary Medicines Authority 2003). Residual study on cow shows that 1% of the total administered boscalid dose was found in milk and tissues (0.43-0.61 mg equiv./kg in liver and 0.06-0.15 mg equiv./kg in milk) and about 90% of the total dose was excreted (Australian Pesticides and Veterinary Medicines Authority 2004).

Even though no residues were found in the offspring, there was an obvious effect of these pesticides on the hormones level and ovarian follicles.

5. Conclusions:

From the results of the present study, the following conclusions can be drawn:

- Under the conditions of the present experiment, there was no, or little impact, of the two pesticides and their mixtures on mother's weight, conceiving ability, number of offspring and weight of offspring.
- Both CYP and SIG showed interference with levels of the four hormones (progesterone, FSH, LH and estradiol) an CYP seems to be a stronger hormonal disruptor than SIG.
- In general, both pesticides and their combinations were found to cause many obvious histopathological disorders in mother and pups ovarian tissues exposed to the higher doses of the pesticides and their mixtures.
- Both pesticides are genotoxic in all their treatments and doses and mixing both insecticide and fungicides together increases their genotoxicity.

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7. Appendices:

Table 1: Mean weights (g) ±SEM of female rats throughout gestation and lactation.

Group	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
C	152.3±5.2	201.5±6.5	241.0±15.0	293.5±23.5	253.5±12.5	268.5±9.5	229.5±17.5
CYP1	161.2±3.0	166.0±1.7	181.7±4.3	244.3±11.4	199.7±17.2	227.7±16.6	202.3±22.8
CYP2	151.4±2.2	170.3±9.9	186.0±11.1	249.7±8.7	208.3±9.7	222.0±28.0	200.0±12.0
SIG1	154.7±6.5	199.0±11.2	216.8±14.0	275.5±10.6	229.0±14.1	223.0±19.9	206.8±21.3
SIG2	166.8±2.7	191.5±8.8	221.3±4.0	270.8±9.3	246.8±5.1	237.8±6.2	210.3±9.6
MIX1	179.0±1.1	249.0±9.0	280.7±12.5	310.7±4.7	252.3±8.4	239.7±25.7	198.0±13.3
MIX2	179.5±0.3	195.5±0.5	232.0±18.0	296.5±8.5	266.5±5.5	262.0±24.0	244.0±23.0

Table 2: Change in weight (g) of female rats throughout the experiment (pregnancy and lactation periods). Values represent means± SEM.

day	C	CYP1	CYP2	SIG1	SIG2	MIX1	MIX2
7	34.0±1.0	14.5±1.5	27.0±5.0	36.7±5.0	16.5±6.3	64.3±6.0	16.0±1.4
14	39.5±8.5	15.7±3.2	15.7±9.9	17.8±7.6	40.0±3.0	31.7±7.2	36.5±17.5
21	52.5±8.5	62.7±7.2	62.7±18.8	58.8±6.3	49.5±5.7	30.0±10.3	64.5±9.5
28	-40.0±11.0	-44.7±6.4	-41.3±5.9	46.5±10.6	-24.0±4.8	-58.3±12.6	-30.0±3.0
35	15.0±3.0	28.0±2.5	15.5±11.5	-6±14.2	-9.0±2.9	-12.7±21.7	-4.5±18.5
42	-39.0±27	-25.3±10.5	-22.0±16.5	-11.7±5.8	-25.5±12.7	-41.7±24.4	-18.0±1.0

Table 3: Pup' weight (g)/group from birth to weaning (day 21). Values represent mean± SEM. N: number of pups. (*): indicates significant difference from the control.

Treatments	Days (N)				Death % at weaning
	0	7	14	21	
C	6.2±0.6 (N=24)	18.1±1.5 (N=13)	33.0±0.6 (N=12)	50.5±0.5 (N=12)	50
CYP1	5.3±0.4 (N=26)	15.1±3.4 (N=23)	26.5±6.6 (N=19)	41.1±10.0 (N=19)	26.9
CYP2	5.2±0.4 (N=44)	14.9±0.6 (N=33)	24.8±2.1 (N=29)	41.4±3.34 (N=29)	34.1
SIG1	5.9±0.6 (N=33)	19.9±1.1 (N=25)	30.8±0.3 (N=22)	50.6±0.4 (N=21)	36.4
SIG2	7.2±0.4 (N=35)	17.7±1.3 (N=33)	27.4±1.8 (N=18)	42.8±0.0 (N=17)	51.4
MIX1	6.6±0.8 (N=30)	13.2±2.4 (N=28)	20.4±2.3 (N=28)	26.3±2.8* (N=27)	10.0
MIX2	6.0±0.2 (N=28)	11.4±1 (N=23)	22.2±2.1 (N=23)	37.5±4.1 (N=23)	23.0

Table 4: Level of different hormones in serum of female rats subjected to pesticides for 42 days compared to the control. Values represent mean±SEM.

Parents	Progesterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	FSH (mlU/ml)
C	116.8±30.0	7.3±0.8	8.8±0.7	1.4±0.4
CYP1	148.6±82.4	6.5±2.7	2.6±0.4	0.8±0.3
CYP2	150.3±38.8	19.0±1.7	0.5±0.3	1.1±0.3
SIG1	199.1±61.5	10.5±1.2	3.0±1.9	3.1±1.2
SIG2	131.3±46.5	8.1±0.6	5.4±1.4	1.1±0.4
MIX1	158.4±57.5	8.7±4.1	3.6±0.9	1.2±0.2
MIX2	119.7±13.7	2.7±0.5	1.3±0.2	1.1±0.5

Table 5: Level of different hormones in serum of female pups subjected to pesticides through their mothers during pregnancy and lactation in comparison with the control. Values represent mean±SEM.

Female Offspring	Progesterone (ng/ml)	Estradiol (pg/ml)	LH (ng/ml)	FSH (mlU/ml)
C	6.2±1.2	2.6±0.4	1.0±0.4	11.6±2.3
CYP1	9.0±0.9	20.7±1.8	1.1±0.2	9.2±1.9
CYP2	10.8±0.7	3.0±0.7	1.1±0.2	1.3±0.3
SIG1	6.6±1.0	6.9±0.6	2.5±1.1	1.3±0.2
SIG2	7.6±0.7	5.8±1.7	2.0±1.0	2.2±0.1
MIX1	6.8±0.5	7.6±0.7	2.5±0.9	0.7±0.2
MIX2	2.8±0.3	2.9±0.8	0.7±0.5	3.6±0.0

Table 6. Total number (MEAN±SEM) of normal follicles in the control mothers and female pups.

Groups	C	CYP2	SIG2	MIX2
Mothers	25.7±4.9	9.3±0.8	12.0±2.0	13.5±4.5
Pups	21.5±1.3	9.0±1.0	20.7±4.1	16.0±2.1

Table 7. Summary of the number (MEAN±SEM) of primary, secondary and Graafian follicles in addition to corpus luteum in ovaries of mothers` groups of four treatments.

Groups	Primary follicle	Secondary follicle	Graafian follicle	Corpus luteum
C	9.0±2.6	3.8±0.3	3.5±0.7	6.3±1.4
CYP2	4.3±0.5	1.0±0.4	0.5±0.3	3.5±0.3
SIG 2	3.5±0.5	3.0±0.0	1.5±0.5	4.0±1.0
MIX2	6.5±3.5	2.5±0.5	3.0±1.0	1.5±0.5

Table 8. Summary of the number (MEAN±SEM) of primary, secondary and Graafian follicles in addition to corpus luteum in ovaries of pups` groups (1 month old) of four treatments.

Groups	Primary follicle	Secondary follicle	Graafian follicle	Corpus luteum
C	9.5±1.3	3.8±0.3	3.5±0.3	4.8±0.9
CYP2	4.7±0.9	1.7±0.3	1.0±0.6	1.7±0.3
SIG 2	17.5±4.7	4.5±1.0	2.5±0.3	0.5±0.3
MIX2	12.0±6.3	2.5±1.0	3.5±0.7	3.0±0.9

Table 9. Atretic follicles percentage (MEAN±SEM).

Groups	C	CYP2	SIG2	MIX2
Mothers	15.5±2.4	27.6±3.3	51.1±5.0	19.8±4.0
Pups	16.8±3.2	42.3±7.7	30.6±6.4	25.5±2.9

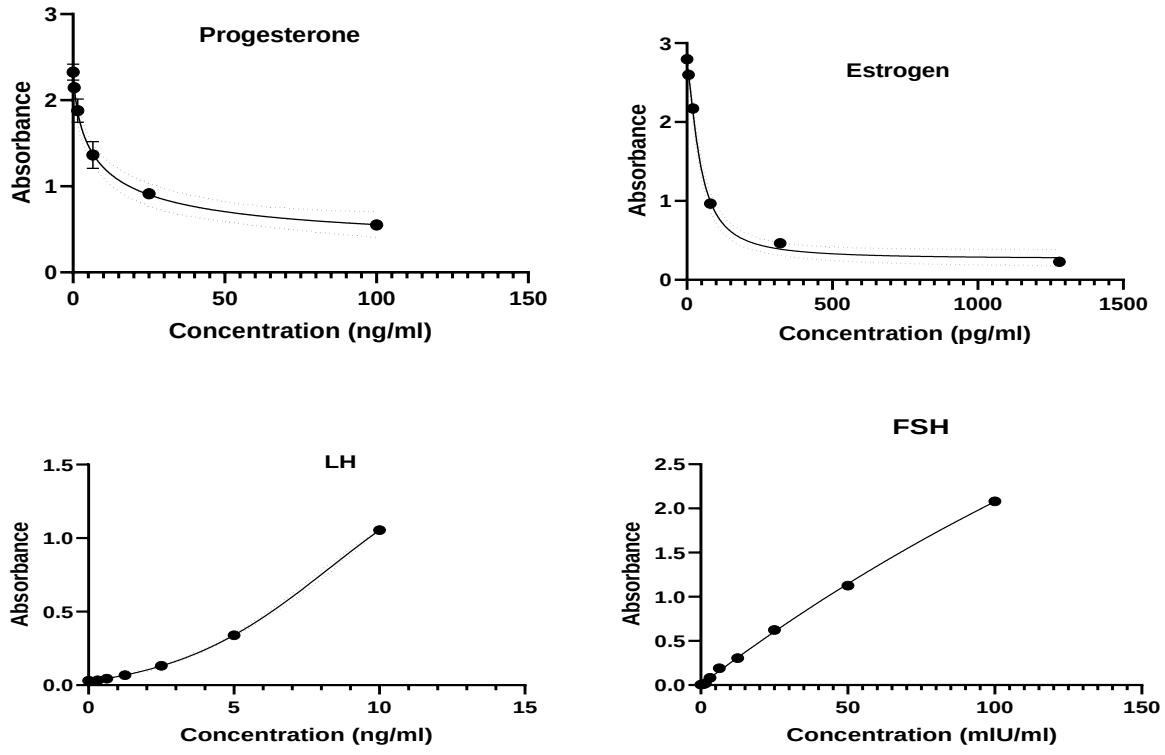

Figure 1: ELISA standard curves with $R^2 \geq 0.99$

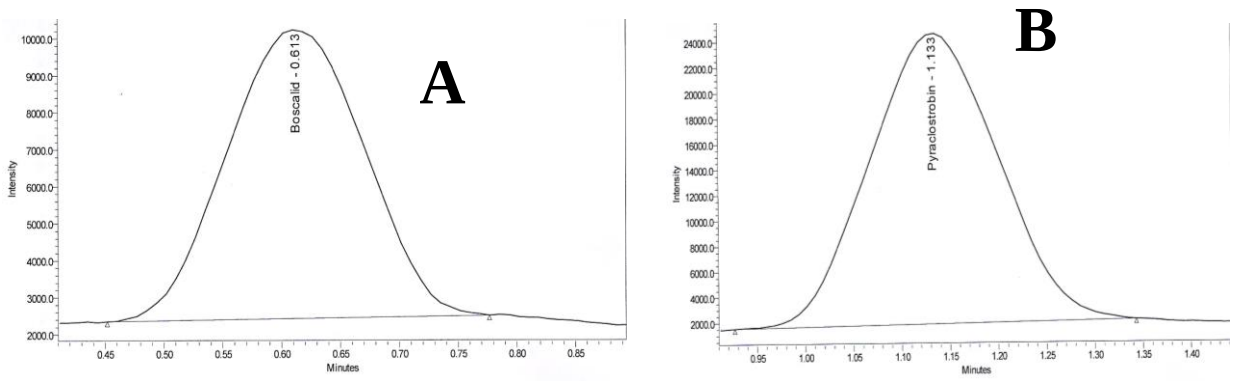

Figure 2: Standard curve for boscalid (A): 216.8 ppb with area under the curve of 0.613 and pyraclostrobin (B): 54.4 ppb with area under the curve 1.133.