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CHROMOSOMAL ABERRATION AND PATHOLOGICAL ALTERATION AFTER COPPER OXYCHLORIDE FUNGICIDE INTOXICATION IN RABBITS

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This study was conducted to examine the effect of Copper oxychloride fungicide is one of the most fungicide used in agriculture fields and crop storage on chromosomal aberration and some pathological alterations on rabbits. Forty five female rabbits divided into three groups (fifteen does each). The 1st group was fed ration without fungicide (control). The 2nd group was fed contaminated ration by Copper oxychloride (0.1184 g /Kg. BW/day as 1/20 LD_{50}) and 3rd group was fed contaminated ration by copper oxychloride 0.2368 g /Kg. BW/day as 1/10 LD_{50} for sixty days. Three does and nine offspring were slaughtered from each group to obtained bone marrow for cytogenetic testing and histopathological examination.

These results significant increase ($P< 0.05$) in total chromosomal aberrations (gap formation, chromosome break, deletion chromosome, centromeric attenuations, centric fusion, end to end associations and ring chromosome) among different groups and among does in the same group and their progeny as fungicide treatment. On the other hand we showed significant increase ($P< 0.05$) in the incidence of clinical signs and congestion of the portal blood vessels with severe inflammation and cellular infiltration of hepatic parenchyma mainly lymphocytes in liver, and high increase congestion of the lining epithelium of renal tubules in the form of cloudy swelling and vacuolar degeneration in kidney in 3rd group more than the other group. The degeneration and atrasia of some ovarian follicles in ovary and increase congestion of the uterine blood vessels, focal areas of endometrial hemorrhages and hyperplasia of the endometrial lining epithelium of uterine horn associated with the increased of copper oxychloride /Kg. BW/day.

***Conclusively**, these results indicated that fed contaminated ration with copper oxyhloride fungicide decreased the reproduction*

performance of female rabbits and make many deleterious effect on animal health.

Key words: Rabbits, Copper, fungicide, chromosomal aberration & pathological.

Copper oxychloride Cu (OH) Cl is one of agriculture fungicide which used commonly to prevent crop damage in the field and protect the harvest crops from deteriorations during storage or transport. There are many agriculture fields intensively applicated because of its low cost and good efficacy (ACP, 2007).

In animals, copper salts have moderate acute toxicity, with soluble salts being more toxic than insoluble ones. The data are limited but rats appear to be more tolerant to acute copper toxicity than other laboratory species Haywood, (1985). In sub chronic repeat dose toxicity tests, copper salts are associated with effects such as gastro-intestinal irritation and liver and kidney toxicity but no observed adverse effect level for copper sulphate of 16 mg/kg body weight/day in a 13-week study in rats (Herbert *et al.,*1993). Copper salts have been reported to have adverse effects on reproductive and developmental parameters like testes, seminal vesicles, uterus and ovaries have been reported at doses of <27 mg/kg body weight/day copper (WHO, 1999& 2006). The rats offspring exposed to greater than 65 mg/kg body weight/day copper reduced fetal weight, size and viability and delayed ossification (Haddad *et al.*, 1991). There are a few adequate data on chronic toxicity of copper salts. There is no evidence of direct carcinogenicity but different copper concentrations appear to have a modifying effect on tumors initiated by other agents (WHO, 2008). However, high levels of copper accumulate in the liver of rat associated with a high incidence of hepatocellular carcinoma (Masuda *et al.*, 1992).

Chromosomal abnormalities can result from either a variation in the chromosome number or from structural changes. These events may occur spontaneously or can be induced by environmental agents such as chemicals, radiation, and ultraviolet light. However, mutations are most likely due to mistakes that occur when the genes are copied as the cells are dividing to produce new cells (Cohen and Jon 2002).

Therefore, a little information is available on the effect of copper oxychloride fungicide on female's administration on some reproductive aspects rabbits taking in consideration the changes that might occur in some relevant blood biochemical values, chromosomal changes and pathological

alterations of genital organs. The objective of this study was to evaluate chromosomal aberration and pathological alteration effect in rabbits.

MATERIALS AND METHODS

Experimental work:

Forty five female rabbits divided into three groups (fifteen does each). The first group (control) was fed uncontaminated ration, the second group was fed contaminated ration with. Copper oxychloride (0.1184 g/Kg. BW/day as 1/20 LD_{50}) and third group was fed contaminated ration with copper oxychloride (0.2368g/Kg. BW/day as 1/10 LD_{50}) for sixty days. Three mothers and nine offspring were slaughtered from each group to obtain bon marrow for cytogenetic according to (Pirtskhelami *et al*., 2008), and testing liver, kidney, ovary and uterine horn for histopathological examination according to Cullig (1974).

Chromosomal analysis:

The animals were injected with 0.025% colchicines at dose of 0.01 ml/g intraperitoneally before slaughtering two hours to block the cells in metaphase. Animals were anesthetized with ether and processed for bone marrow sampling using the method described by Rabello-Gay and Ahmed (1980). The cells in bone marrow were flushed by pushing 2-3 times (0.9%NaCl) into the marrow cavity of femur and tibia. Cells were collected, left in (0.56% KCl) for 20 min in incubator at 37c˚ and centrifuged at 1000 rpm for 8 min. The supernatant was removed; the pellet was resuspended and fixed in 5 ml of methanol: acetic acid (3:1) for 20 min. Repeat the centrifugation and fixation two or three times. Finally, the cells were dropped on a clean wet slide and uniformly stained using a 5% Giemsa stain. The classification of aberrations was carried out as described by (Venitt and Parry 1984) and in the International System for Cytogenetic Nomenclature (ISCN 1985) From each slide 50 cells were scored under 1000x magnification to determine the frequencies of cell chromosome damaged. Aberrations were pooled as described by (Wise et al.1994). This is because deletions can only be unequivocally distinguished from achromatic lesions if the distal from centric fusion is displaced. Thus, pooling aberrations avoids artificial discrepancies between scorers due to different perceptions of the width of an achromatic lesion relative to the width of its chromatid (Wise et al.1994). Accordingly, chromatid deletions and achromatic lesions were pooled as chromatid lesions. The frequency of chromosome aberrations was estimated on 50 metaphases.

Histopathological examination:

The organs (liver, kidney, ovary and uterine horn) were immediately removed and washed in saline solution. The organs were fixed in 10% phosphate buffered formalin. Following an overnight fixation, the specimens were dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax (58-60 C). Blocks were made and sectioned of 5 µm thickness with microtome. The tissue sections were stained with hematoxylin and eosin and observed under the light microscope.

Statistical analysis:

Analyses of variance (ANOVA) were carried out using the SAS software package (2008). Duncan's new multiple range tests were used to test the significance of the differences among means (Duncan, 1955).

RESULTS AND DISCUSSION

Chromosomal Investigation:

Sixty days of feeding forty five female rabbits on contaminated ration with two levels of Copper oxychloride (0.1184 g/Kg. BW/day as 1/20 LD_{50}) and (0.2368g/Kg. BW/day as 1/10 LD_{50}) were tested compared with the control group. The increased of the chromosomal aberrations were associated with the increased of the concentration of the copper oxychloride fungicide. We observed significant different among groups in NZW rabbits as shown in (Table 1) and the contamination with the fungicide induced significant increase ($P < 0.05$) in total chromosomal aberrations among does and their offspring (Table 2).It may be due to accumulation of the heavy metals (copper oxychloride) in deferent tissues lead to damage of DNA as result of treatment. These results are in agreement with Alfredo Corona-Rivera et al., (2007) who observed a statistically significant increase of DNA damage in individuals exposed to 390 µg/ml of copper intake and Pirtskhelani et al., (2008), who found that oral dosage of copper oxychloride (1/2, 1/5, 1/10 LD50) in white mice induce significant increase ($p<0,001$) of chromosomal aberrations frequency (multiple fragments, lyses), genomic mutations (triploidy, tetraploidy) pathological mitosis (hollow metaphase, K-mitosis, adhesion of chromosomes) and destruction of interphase nucleuses (hollow nucleus).

Table1: Chromosomal structural aberration as affect by copper oxychloride fungicide ($\bar{x} \pm$ se).

Chromosomal Structural aberrations	**Treatments mg Cu/ kg/Bw/day**		
	Control	**0.1184 gm**	**0.2368 gm**
Gap	0.04 ± 0.18^{b}	0.38 ± 0.18^{b}	1.42 ± 0.18^{a}
Break	0.13 ± 0.16	0.42 ± 0.16	0.42 ± 0.16
Deletion	0.83 ± 0.30^{b}	2.92 ± 0.30^{a}	2.88 ± 0.30^{a}
Centromeric Attenuation	0.38 ± 0.22^{b}	0.88 ± 0.22^{a}	1.38 ± 0.22^{a}
Centric fusion	0.54 ± 0.19	0.63 ± 0.19	0.88 ± 0.19
End to end	0.33 ± 0.13^{b}	0.13 ± 0.13^{b}	0.58 ± 0.13^{a}
Ring	0.17 ± 0.20^{b}	0.88 ± 0.20^{a}	1.21 ± 0.20^{a}
Sticky	0.25 ± 0.20^{c}	1.63 ± 0.20^{b}	2.17 ± 0.20^{a}
Total	**2.71 ± 0.50^{c}**	**8.75 ± 0.50^{b}**	**12.17 ± 0.50^{a}**

Values with different letters in the same row are significantly different ($P<0.05$).
50 cells were examined per animal.

The significant induction of chromosomal/ mitotic aberrations exposed to chromium compounds in present study indicated its genotoxic potential. The aberrant mitotic stages may have been the outcome of spindle poisoning that causes chromosome disturbances during mitotic cell division. Various heavy metals are known to induce chromosome breaks, fragments and micronucleus formation in plants and mammalian test systems (Knasmuller *et al.*, 1998), and their effects were emphasized to be the result of formation of DNA-DNA and DNA-protein cross-links.

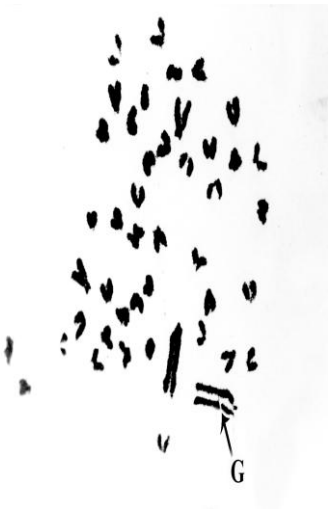
In (Figure 1) we showed the most prevalent types of aberrations were gap formation (the lost area is shorter than diameter of chromatid), chromosome break (the lost area is longer than the diameter of chromatid or equal to it), deletion chromosome (one chromatid short than its sister), centromeric attenuations (the arm of one chromatid was separated from its sister), centric fusion (the one chromosome is fusion with another chromosome by centromers), end to end associations (two chromosomes were joined at the proximal ends of the two chromatids) and ring chromosome (the ends of the two chromosome's arms were joined together) as a results to copper oxychloride fungicide treatment.

Wise *et al*., 1994 and Stearns *et al.*, 1995, reported that various types of DNA damage occur in response to exposure to heavy metals (chromium compounds) including DNA single strand breaks, DNA-protein crosslinks,

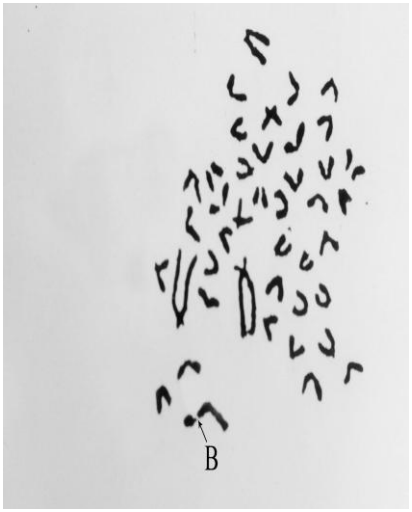
Table2: The difference responded between the does and offspring to the effect of copper oxychloride fungicide on chromosomal structural aberration ($\bar{x}$ ± se).

Chromosomal Structural aberrations	**Type /No**	
	Doe /9	**Offspring/27**
Gap	0.92 ± 0.18[a]	0.31 ± 0.10[b]
Break	0.25 ± 0.16	0.29 ± 0.09
Deletion	2.42 ± 0.03	2.00 ± 0.17
Centromeric Attenuation	1.08 ± 0.22	0.87 ± 0.13
Centric fusion	0.83 ± 0.19	0.53 ± 0.11
End to end	0.33 ± 0.13	0.36 ± 0.08
Ring	0.67 ± 0.20	0.83 ±.0.10
Sticky	1.75 ± 0.20[a]	0.94 ± 0.10[b]
Total	**9.33 ± 0.5[a]**	**6.42 ± 0.3[b]**

Values with different letters in the same row are significantly different (P<*0.05*). 50 cells were examined per animal.

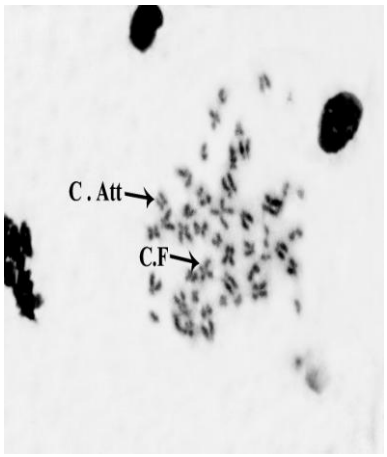


(a)



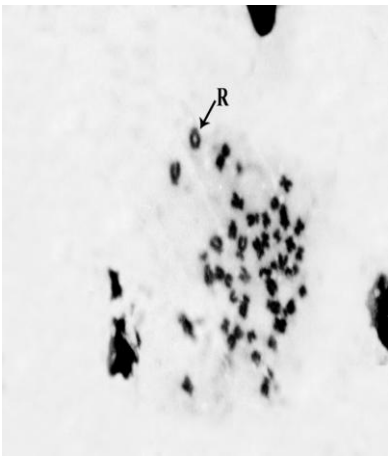
(b)

(c)



(d)

(e)



(f)

(Figure1): Metaphase spread of NZW rabbits fed on contaminated ration with copper oxychloride fungicide, showing (a): gap, (b): break, (c): deletion, (d): centromeric attenuation and centric fusion, (e): end to end and (f): ring.

Cr-DNA adducts, and DNA-DNA crosslinks. Sugiyama *et al*., 1991 and Manning *et al.*, 1992, found the pathways for DNA damage by heavy metals in the presence of biological reducing agents appear to be highly complex. (Sugiyama *et al.*,1991 and Manning *et al.*, 1992).

Histopathological changes:

The microscopic examination of the liver of rabbits fed ration contaminated with copper oxychloride (0.1184 g, Cu (OH) Cl. /kg.BW/day) as

1/20 LD50 for 60 days- revealed slight congestion of the portal blood vessels with hydropic degeneration of hepatocytes (H.D.H) as shown in Figure 2a. Also, samples in the third group (0.2368g Cu (OH)Cl /kg.BW/day) as 1/10 LD50, showed hydropic degeneration of hepatocytes with inflammatory cellular infiltration of the hepatic parenchyma mainly lymphocytes. These results are in agreement with Haywood (1979) and Hore *et al.*(1997) who recorded that the signs of copper toxicity due to the most highly supplement diets in rate were early liver damage and pathological changes significantly and Singh KK. et al., (2010) who showed significant an increase in Cu concentration in the liver when recorded in fenvalerate intoxication of goats given orally 15mg Cu/kg./day for 270 days, and Alfredo Corona-Rivera *et al*., (2007) who observed a statistically significant damage increase in individuals exposed to 390 µg/ml of copper.

The histopathological changes due to copper toxicity in the kidney of rabbits were appeared in multiple areas as cloudy swelling of the lining epithelium (C.S.L.E) of some renal convoluted tubules. These microscopical changes were observed in the examined kidney of rabbits that were fed on contaminated ration with copper oxychloride (0.1184 g Cu (OH) Cl /kg. BW/day) as 1/20 LD50 for 60 days (the second group). But in the third group fed on (0.2368g Cu (OH) Cl /kg .BW/day) in fed as 1/10 LD50 for 60 days showed congestion of the lining epithelium of renal tubules in the form of cloudy selling and vacublar degeneration (Figure 2 b). These results agreed with those observed by Haywood (1985) and Hore *et al.* (1997) who showed that the pathological significant changes in the kidney of rats and Abdel-Hafez (1995) who observed, in NZW rabbits, that the kidneys were soft and there were a pathological changes in its tissues.

The trace elements of the fungicide produced considerable changes of the ovarian follicular structures and the corpora lutea. The interrelationships of morphological and functional changes with increased occurrences of trace elements in various tissues necessitate further investigation.

In the present study the microscopical examination of rabbit ovaries in the third group that given (0.2368g Cu (OH) Cl /kg. BW/day) as 1/10 LD50 for 60 days revealed histological changes degeneration and atrasia of some ovarian follicles (Figure 2 c). These results are in agreement with Bires *et al.* (1995) who showed, in sheep that fed diet contaminated with copper intoxication, the histological changes in number of ovarian follicles and increased occurrence of primary atretic follicles and Ahmed *et al.* (1998) showed that copper treated rats revealed absence of mature grafian follicles and decreased number of growing follicles. Niswender *et al.* (1985), Wolfenson &Orlyblum (1988) and Kliment & Zithy (1989) who showed that in rabbits treated with copper small and large follicles which originate from granulose and theca cells.

Congestion of the uterine blood vessels- focal areas of endometrial hemorrhages and hyperplasia of the endometrial lining epithelium were seen in the examined uterine horn of treated rabbits (Figure2 d). These results agreed with finding of Bires et al.(1995) who showed that in ewes fed on high level copper diets, the copper (Cu) was concentrated in uteri and changes pathological endometrium and Ahmed *et al.* (1998) who showed that microscopical examination of uterus of copper treated female rats – the surface epithelial cells showed focal hyperplasia- uterine glands showed degenerative and necrotizing changes of their epithelium lining.

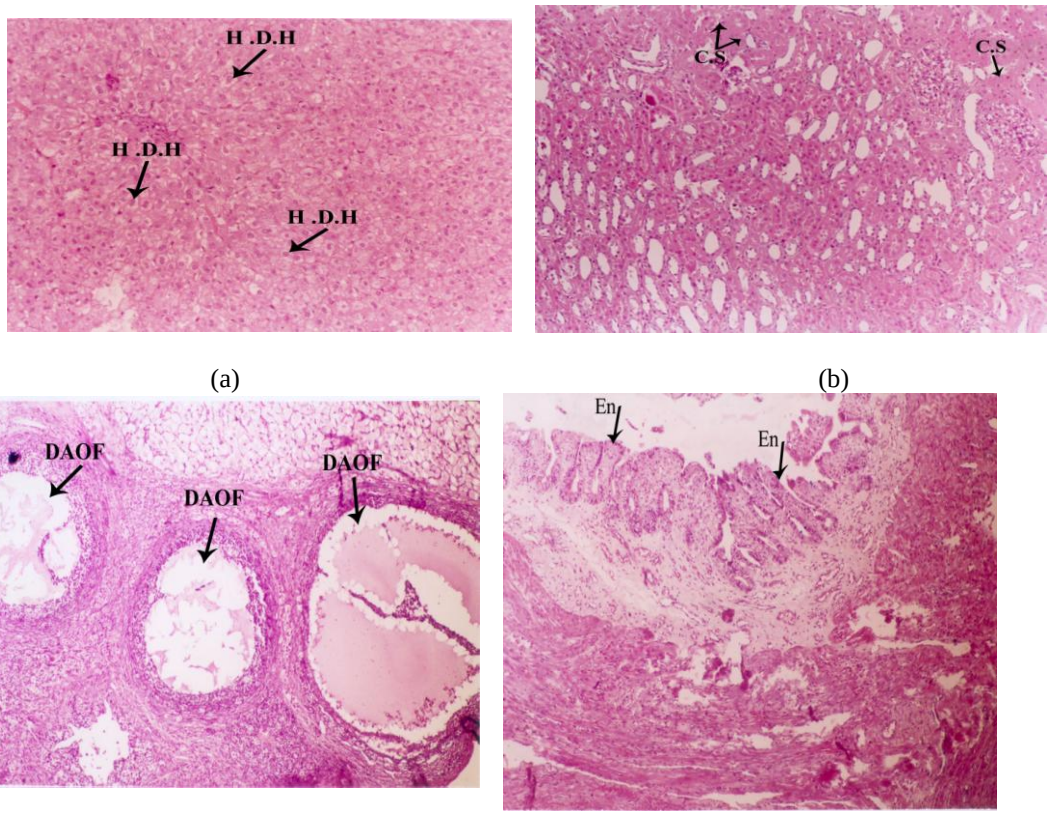


Figure 2: (a) Liver of rabbits fed on ration contaminated with fungicide, showing extensive hydropic degeneration of hepatocytes (H.D.H) and congestion of central veins (C.V). (b) : Kidney showing cloudy swelling of the lining epithelium of renal convoluted (C.S.L.E) tubules. (c) :Ovary, showing increased number of atretic follicles. (H&E X200) and (d): Endometriun, showing focal hyperplasia of the epithelium lining. (H&EX200) arrows.

Conclusively, it was concluded that, due to toxicity of copper oxychloride and the widely using of this compound in agriculture, the use of

this compound must be restricted as been as possible. Liver and kidneys of copper oxychloride exposed animals were contained with the highest copper residue levels. Copper oxychloride fungicide is the etiological factors to chromosomal aberrations, which lead to mutagenic, teratogenic and carcinogenic effect on long term contamination of human food and animal feed. It also, produces irreversible damage to some internal organs such as liver and kidney. Periodical examination should be done for meat and other meat products as well as the edible organs and their load for heavy metals which also should be evaluated according to the international guide lines as a fruitful advice to delay environmental contamination.

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التغيرات الكروموسومية و الأعراض المرضية الناتجة عن أستخدام المبيد الفطري أكسي كلورو النحاس السمي علي الأرانب

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أجرى هذا البحث لدراسة تأثير تلوث مواد العلف المختلفة (الأعلاف الخضراء والمصنعة) بالمبيدات الفطرية مثل (أوكسى كلورو النحاس) على حيوانات المزرعة حيث استخدم فى هذه الدراسة عدد ٤٥ أنثى ناضجة من الأرانب النيوزيلاندى الأبيض فى عمر ٥-٦ شهور بمتوسط وزن ٣ كجم وقد تم تقسيمها الى ٣ مجموعات الأولى منها ضابطة وهذه غذيت على عليقة مصنعة خالية تماما من أى تلوث بالمبيد ، والمجموعة الثانية غذيت على عليقة ملوثة بالمبيد بواقع ٠,١١٨٤ جم من المبيد/ /كجم وزن حى للحيوان / يوميا (٢٠/١ من الجرعة النصف مميتة)، أما المجموعة الثالثة فقد غذيت على عليقة ملوثة بالمبيد بواقع ٠,٢٣٦٨جم / كجم وزن حى للحيوان/ يوميا أى حوالى١(/١٠ من الجرعة النصف مميتة) وكانت نسبة تركيز عنصر النحاس فى هذا المبيد الفطرى حوالى ٥٠% وقد استمرت التغذية لمدة ٦٠ يوما٠

وبعد انتهاء مدة التغذية تم ذبح ٣ أمهات من كل مجموعة وكذا ذبح عدد ٣ أبناء لكل أم أى أن العدد الكلى للحيوانات المذبوحة ٢٧ حيوان لدراسة التغيرات السيتوجينية والهستوباثولوجية .

أولا : الدراسة السيتوجينية :

تم فحص ٤٥٠ طور استوائى للأمهات و ١٣٥٠ طور استوائى للنسل فأوضحت الدراسة أن نسبة الاختلالات فى التراكيب الكروموسومية كانت ٢,٧١% ، ٨,٧٥% ، ١٢,١٧% فى المجموعات الثلاثة على الترتيب كما أوضحت الدراسة أن نسبة الاختلالات الكروموسومية فى النسل كانت ٦,٤٢%.

مما سبق يتضح أن هناك زيادة معنوية فى نسبة الاختلالات الكروموسومية نتيجة التعرض للتركيزات المختلفة من المبيد الفطرى بين المجاميع وأن هناك تناسبا طرديا بين تركيز المبيد المستخدم وبين نسبة الاختلالات الكروموسومية للأفراد.

ثانيا :الدراسة الهستوباثولوجية:

تم أخذ عينات لكل من الأعضاء (الكبد – الكلية – المبيض – الرحم) للدراسة الهستوباثولوجية حيث أنه من خلال الفحص الهستوباثولوجى تلاحظ وجود اصابة سائدة بالخلايا الكبدية ومعظمها بؤرى ويصاحبها أحيانا تفاعل خلوى للخلايا

وحيدة النواة وذلك فى المجموعة الثانية . كماشوهد أيضا فى المجموعة الثالثة زيادة فى تليف الخلايا الكبدية وزيادة فى التهاب بعض المناطق مصحوبا بنزيف دموى فى نسيج الكلية وكانت خلايا الأنابيب الكلوية منتفخة وبها حبيبات واحتقان فى المنطقة المبطنة لجدر الأنابيب الكلوية وذلك فى كل من الجموعتين الثانية والثالثة ، كما لوحظ عند فحص المبيض أن هناك زيادة معنوية فى ضمور فى الحويصلات المبيضية الأولية والثانوية وذلك فى الجموعتين الثانية والثالثة كما شوهد أيضا فى نفس المجموعتين زيادة معنوية فى احتقان الأوردة الدموية المغذية للرحم مع وجود التهابات فى النسيج الحمى وتضخم الغدد الرحمية وغزو لخلايا مستديرة للطبقة المبطنة للرحم.

التوصية: يوصى البحث مراعاة عدم تعرض حيوانات المزرعة لأى تلوث من المبيدات المستخدمة سواء فى الانتاج الزراعى أو الانتاج الحيوانى مع أخذ الاحتياطات الكافية لتجنب هذه الملوثات كما يوصى البحث العمل على استخدام البدائل العضوية فى مكافحة الفطريات فى الانتاج الزراعى. كما أنه يوصى بعدم أكل بعض الأعضاء الداخلية للذبيحة واستبعادها مثل الكبد والكلية .