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TOXICITY EVALUATION OF SOME PHYTOSANITARY PRODUCTS WIDELY USED IN THE FIELD OF AGRICULTURE IN ALGERIA ON LABORATORY ANIMALS

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...DEDICATION...

*To my beloved **PARENTS***

Abstract

This study aims to evaluate the toxic effects of the used fungicides Methyl Thiophanate (MT) and Thiram on fertility, hematological, biochemical, hormonal parameters and the histology of some organs in the male rabbits.

The different groups of animals (6 in each group) was treated with MT by doses (50, 100 and 150 mg / kg) for 4 weeks and Thiram with doses (20, 30 and 80 mg / kg) for 2 weeks by orally system, with a control group.

The results indicate a rate of 100% of rabbits mortality in the group treated with a high dose of Thiram after only two weeks.

The most important results have revealed that both fungicides may induce reprotoxic effects, as shown by the decrease in testes and epididymis weight with histological alterations, with a decrease in the indicators of male fertility (count, motility, speed and viability of sperm) associated with a decrease in testosterone level in the treated groups compared with the control one.

Concerning the data of the effects of MT in the hematological parameters, we noted a decrease in the number of red blood cells, hemoglobin, hematocrit, white blood cells, platelet, MCV and MCHC and an increase of MCH in the treated groups compared with the control one.

The treatment with Thiram for 2 weeks decreased the concentration of RBC, HCT, PLT, MCV and MCHC, while the concentration of WBC, HGB and MCH increased in the treated groups compared with the control group.

The results of biochemical parameters revealed a perturbation in the levels of glucose, triglyceride, cholesterol, urea, uric acid and albumin. And a decrease in Thyroids hormones T3 and T4 also observed after administration of both fungicides (MT and Thiram) compared with the control group.

Concerning the histological study the results show that the treatment with the used doses of MT and Thiram caused a damage of hepatocyte tissue revealed by necrosis, cytoplasmic vacuolization and dilatation of blood vessels with an increase in liver weight. In addition the observation of the microphotographs of renal tissue in the treated animals showed

histological changes revealed by impairment of tubular and vascular accompanied by degeneration of renal glomerulus as well as a decrease in kidney weight.

In conclusion, the treatment with the fungicides MT and Thiram in the same experimental conditions may affect many biological markers especially the fertility in male rabbits.

Keywords: Male Fertility, Rabbit, Toxicity, Methyl Thiophanate, Thiram, Physiology Histopathology.

Résumé

Cette étude vise à évaluer les effets toxiques des fongicides utilisés (Thiophanate Methyl et Thirame) sur la fertilité, l'hématologie, les paramètres biochimique, hormonale et l'histologie des organes chez le lapin mâle.

Les différents groupes d'animaux ont été traités par Thiophanate Methyl aux doses (50, 100 et 150 mg / kg) durant 4 semaines et le Thiram aux doses de (20, 30 et 80 mg/kg) pendant 2 semaines.

Les résultats indiquent un taux de mortalité de 100% chez le groupe traité à la dose la plus élevée de Thiram seulement après 2 semaines.

Les résultats les plus importants ont révélé que: les deux fongicides, peuvent induisent des effets reprotoxiques révélés par une diminution de la masse du testicules et l' épididymes avec des altération histologique accompagnées d'une diminution des indicateurs de la fertilité masculine (la concentration, la motilité, la vitesse et la viabilité des spermatozoïdes) avec une diminution de la concentration de testostérone comparés au groupe non traité.

Les résultats montrent également le traitement au TM pendant 4 semaines a provoqué une diminution du nombre des globules rouges, de l'hémoglobine, de l'hematocrite, des globules blancs, des plaquettes, du MCV et de la MCHC et une augmentation de MCH chez les groupes traités par rapport au groupe témoin.

En outre, le traitement avec le Thiram pendant 2 semaines a provoqué la diminution de la concentration de RBC, HCT, PLT, MCV et MCHC, alors que la concentration du WBC, HGB et MCH a augmentée chez les groupes traités par rapport au témoin.

L'étude biochimique a révélé une perturbation dans la concentration des paramètres biochimiques étudiés (glucose, triglycérides, cholestérol, urée, acide urique et albumine) et ainsi que dans les hormones thyroïdiennes T3 et T4) après l'administration des deux fongicides (TM et Thirame).

L'étude histologique a montré que le traitement aux fongicides utilisés a causé hépatotoxicité révélée par la nécrose, vacuolisation cytoplasmique et les vaisseaux sanguins dilatés et congestionnés, accompagnée d'une augmentation de la masse du foie. L'observation des coupes histologiques du rein chez les animaux traités ils ont induit des changements histologiques (une insuffisance vasculaire avec une dégénérescence du glomérule rénal) accompagné d'une diminution de la masse du rein.

Nous Concluons que le traitement aux deux fongicides dans les mêmes conditions expérimentales peut affecter plusieurs marqueurs biologiques et surtout la fertilité chez le lapin male

Mots-clés: Fertilité Masculine, Lapin, Toxicité, Thiophanate Méthyl, Thirame, Physiologie, Histopathologie.

الملخص

تهدف هذه الدراسة إلى تقييم الأثار السمية لمبيدات الفطريات (ميتيل تيوفانات و التيرام) على بعض مؤشرات الخصوبة, مؤشرات الدم ,المؤشرات البيوكيميائية , الهرمونات و الأنسجة لدى ذكور الأرانب.

عولجت الأرانب بجرعات متفاوتة (50 , 100 و 150 مغ/كغ) من تيوفانات ميتيل لمدة 4 أسابيع و بجرعات (20, 30 و80 مغ/كغ) من التيرام لمدة أسبوعين عن طريق الفم.

أوضحت النتائج نسبة وفيات مرتفعة 100% في المجموعة المعالجة بأعلى جرعة من التيرام.

اتضح أن مبيدات الفطريات المستخدمة في هذه الدراسة أثرت على وزن الخصي و البربخ كما سجلنا انخفاض في بعض المؤشرات الحيوية للحيوانات المنوية (التركيز, الحركة, السرعة و حيوية الحيوانات المنوية), تغيرات نسيجية في الخصيتين و البربخ و إنخفاض هرمون التستوستيرون مقارنة بمجموعة الشاهد.

أظهرت النتائج أن ميتيل تيوفانات له تأثير على مؤشرات الدم حيث سجلنا إنخفاض في تركيز الكريات الدموية الحمراء, تركيز خضاب الدم, الهيماتوكريت, تركيز الكريات الدموية البيضاء, عدد الصفائح الدموية, MCV وMCHC و زيادة في MCH عند المجموعات المعالجة مقارنة بمجموعة الشاهد.

إضافة إلى ذلك، المعاملة بالثيرام لمدة أسبوعين تسببت في إنخفاض تركيز الكريات الدموية الحمراء الهيماتوكريت ، الصفائح الدموية MCV وMCHC بالمقابل لاحظنا زيادة تركيز الكريات الدموية البيضاء، تركيز خضاب الدم و MCH عند المجموعات المعالجة مقارنة بمجموعة الشاهد.

في ما يخص القياسات البيوكيميائية والهرمونية, سجلت تغيرات في بعض القياسات لدى المجموعات المعالجة بميتيل تيوفانات والثيرام عن طريق زيادة تركيز الجلوكوز، الدهون الثلاثية ,الكوليسترول، اليوريا، حمض اليوريك. و إنخفاض تركيز الألبومين , والهرمونات الدرقية T3 وT4 .

سجلت تغيرات نسيجية معتبرة على مستوى الكبد و الكلى للمجموعات المعالجة بكلى المبيدين المتمثلة في النخر، ظهور فجوات حشوية وتوسع الأوعية الدموية وزيادة أوزان الكبد. كما أدت إلى إنخفاض في وزن الكلى و تغييرات في البنية النسيجية المتمثلة في قصور الأوعية الدموية و تدهور بنية الكبيبة الكلوية.

في الختام نستنتج أن المعالجة بالمبيدات المستخدمة في هذه الدراسة لها آثار سامة على القياسات البيولوجية خاصة الخصوبة عند ذكور الأرانب.

الكلمات المفتاحية: الخصوبة، ذكور الأرانب، السمية، ميثيل تيوفانات، ثيرام، وظائف الأعضاء.

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LIST OF ABBREVIATIONS

ADP : Adenosine-5-Diphosphate

Bw : Body Weight

CAS : Chemical Abstracts Service

CHE : Cholesterol esters

DAP : Dihydroxyacetone Phosphate

EDTA : Ethylene Diamine-Tetraacetic Acid-

EPA : Environmental Protection Agency

FSH : Follicle-Stimulating Hormone

GOD : Glucose Oxidase

G3P : Glycerol-3-phosphate

GPO : Dihydroxyacetone Phosphate

GLDH : Glutamate Dehydrogenase

HGB : Haemoglobin

HCT : Hematocrite

H&E : Hematoxylin and Eosin

LD50 : Lethal Dose

LH : luteinizing hormone

LPL : Liberate Glycerol

MT : Methyl Thiophanate

MBC : Carbendazime

MCV : Mean Corpuscular Volume

MCH : Mean Corpuscular Hemoglobin

MCHC : Mean Corpuscular Hemoglobin Concentration

Mg / kg : Milligram / kilogram

Mm Hg : Millimeters of mercury

NADH : Nicotinamide Adenine Dinucleotide Hydrogen

NAD : Nicotinamide Adenine Dinucleotide

PLT : Platelet Count

Ppm : Parts per million

POD : Peroxidase

P : Probability value

RBC : Red Blood Cells

RT : Reagent

SD : Deviation Standard

SPZ : Spermatozoa

T3 : Tri-Iodothyronine

T4 : Thyroxin

µl : Micro Liter

WBC : White Blood Cell

WHO : World Health Organization

$\bar{x}$: Mean

SUMMARY

4. DISCUSSION

Introduction

INTRODUCTION

Phytosanitary products have been used in modern agriculture for a while of time that causes big concerns about the health, particularly in the reproductive system. These products are wide spread worldwide, the fact that makes a difficulty to remove them from agriculture in the near future. Pesticide risks may be greater in developing countries where there are bad conditions of workplaces and safety for workers who are out the priority of the authorities.

Many experimental studies and some observations have suggested that alterations of the male reproductive functions especially semen characteristics are an important health problem all over the world. This may be due to toxic factors present in our environment which particularly involves molecules originally industrial or anthropogenic grouped under the generic term endocrine disruptor, one of the most famous of which are pesticides **(Ferreira, 2010; Hossain et *al*., 2010)**.

These pesticides can alter estrogens functions (Xenoestrogenic), androgens, thyroidien hormones, and even the pituitary gland hormones. Indeed, Endocrine disruptors interact with the endocrine system; it is a complex system composed of many organs scattered around the Organisms (pancreas, adrenal, testes, ovaries, thyroid and parathyroid). Each body secretes hormones released into the body through the bloodstream. The functioning of the endocrine system and strict internal control involved in homeostasis, or biological balance necessary for life. These organs are under control of regulator substances or releasing Factors (hypothalamus) and stimulines substances (pituitary) **(Ferreira, 2010).**

There are many possible ways in which humans can be exposed to the toxic effects of these pesticides may have consequences from food consumers, production workers, formulators, farmers and other applicators. They are widely applied as insecticides, herbicides, and fungicides **(Bozdogan, 2014).**

Fungicides property is to monitor, repel or destroy the fungi that may develop on crops. They are used against fungal diseases such as mildew, powdery mildew and mold. On the other hand, some of the fungicides have shown toxicity to humans, animals, and useful plants, in addition to their persistence (long life) in the environment. Long half-life of chemical in the environment equal a more likely to be exposed to it by the target organisms

and it accumulates making a hazardous dose. Moreover, these chemicals were shown to be present in fruit products prepared for human consumption **(Cabras et *al*., 2000).**

Recently, it has been confirmed that reprotoxic effects of fungicides decreases the sperm quality and in other disorders of the male reproductive tract **(Mallem et *al*., 2007; Gallo and Tosti, 2015).** These fungicides are known to interfere with spermatogenesis by damaging the testes **(Kristensen, 1999)**. The severity of affection that depends on the stage of differentiation can be reversible or irreversible and may even temporarily lead to decrease fertility by modifying sperm count, structure motility, or viability of spermatozoa. These effects are transient after removal of the offending chemical; the spermatogenesis can be restored from stem cell populations **(Bretveld et *al*., 2007).** In fact, normal fertility is based on normal spermatogenesis. In the complex process of spermatogenesis, disturbance can easily occur and result in decreased or absent spermatogenesis and sub-infertility, as a consequence. Assessment of the semen parameters is considered a key indicator in male reproductive capacity, by identificating the adverse effects of the related toxicants on fertility **(Oliva et *al*., 2001; Mehrpour et *al*., 2014).**

Two fungicides are the most widely used in the field of agriculture in Algeria namely: Methyl Thiophanate from Benzimidazole class and Thiram from Dithiocarbamate.

Methyl Thiophanate is generally metabolized to Carbandazim **(Roberts et *al*., 1998; Cardone, 2012).** Both Methyl Thiophanate and Carbendazim have been reported to be capable of causing endocrine disruption, embryotoxic and teratogenic effects **(Yang et *al*., 2011)**, Carbendazim is poorly catabolized and remains in tissues such as gonads, liver, adrenals, adipose tissue, skin and other organs **(WHO, 1993).** It is a well-recognized testicular toxicant **(Yu et *al*., 2009).**

Evidences available support the deleterious effects of Methyl Thiophanate and its metabolite Carbendazim on various aspects of male reproduction in hamsters, rats, rabbits and humans. The effects include the decrease of average testes weight, average seminiferous tubular diameter **(Carter et *al*., 1987)** total sperm count, motility, increased incidence of sperm abnormalities **(Akbarsha et *al*., 2001)** and disruption of microtubule formation **(Nakai et *al*., 2002)**.

Thiram is moderately toxic by ingestion, but it is highly toxic if inhaled. Symptoms of chronic exposure to Thiram in humans include drowsiness, confusion, loss of sex drive,

incoordination, slurred speech and weakness. It has been reported to have adverse effects on the hepatic system**, (Dalvi, 1986)** the reproductive system and on the developmental processes **(Hemavathi et *al*., 1993; Mishra et *al*., 1998).** Studies have shown that Dithiocarbamate decreases the quality of spermatozoa and affects male fertility and Thiram interfere with the differentiation process of spermatogenic cells **(Agrawal et *al*., 1997)**

Objectives:

The objective of this study is to experimentally evaluate the toxicity consequences of the used fungicides Methyl Thiophante and Thiram on the bio-indicator parameters in male rabbits and mainly their effect on male fertility by:

- ✓ Evaluation of some male reproductive parameters.
- ✓ Determination of the concentration of thyroid hormones.
- ✓ Evaluation of some biochemical and hematological parameters.
- ✓ Determination of histological study of some organs.

Review of Literature

1. REVIEW OF LITERATURE

1.1. Definition of Fungicides

Fungicides are a specific type of pesticides that controls fungal disease specifically inhibit or kill fungi causing important diseases (mildew and mold) **(Mc-Grath, 2004)**. Understanding mechanisms of fungicide actions and toxicity is important because humans and domesticated animals encounter these pesticides through a wide variety of applications. In agriculture, fungicides are used to protect tubers, fruits and vegetables during storage. They are also applied directly to ornamental plants, trees, field crops, cereals and turf grasses (**Gupta and Aggarwal, 2007**).

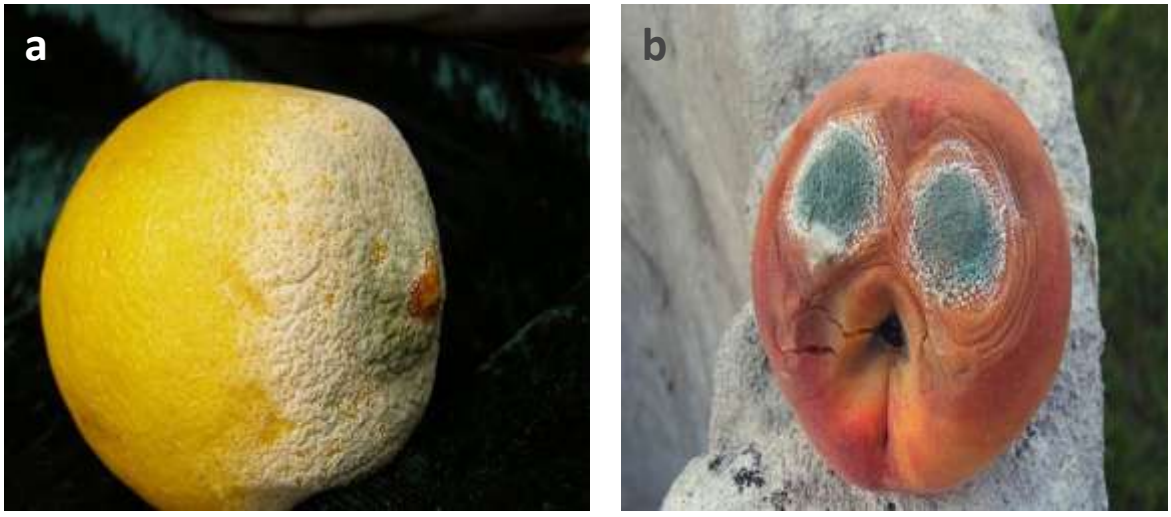


Figure 1. Fungal diseases/ **a**: mildew, **b**: mold **(Anon., 2007)**.

1.2. Fate Processes of Fungicides in Environment

For nearly fifty years, fungicides (generally all pesticides) have been highlighted in all environmental compartments. As well in river waters, groundwater, air, rainwater in addition to the fruits, vegetables and grains. Failure to comply with good agricultural practices can result in contamination of three biosphere compartments namely water, soil and air. Thus, the geochemical cycle of pesticides is very complex for they can be found at all levels.

The following simplified figure shows the various possible uses and drifts of pesticides **(El Mrabet et *al.*, 2008).**

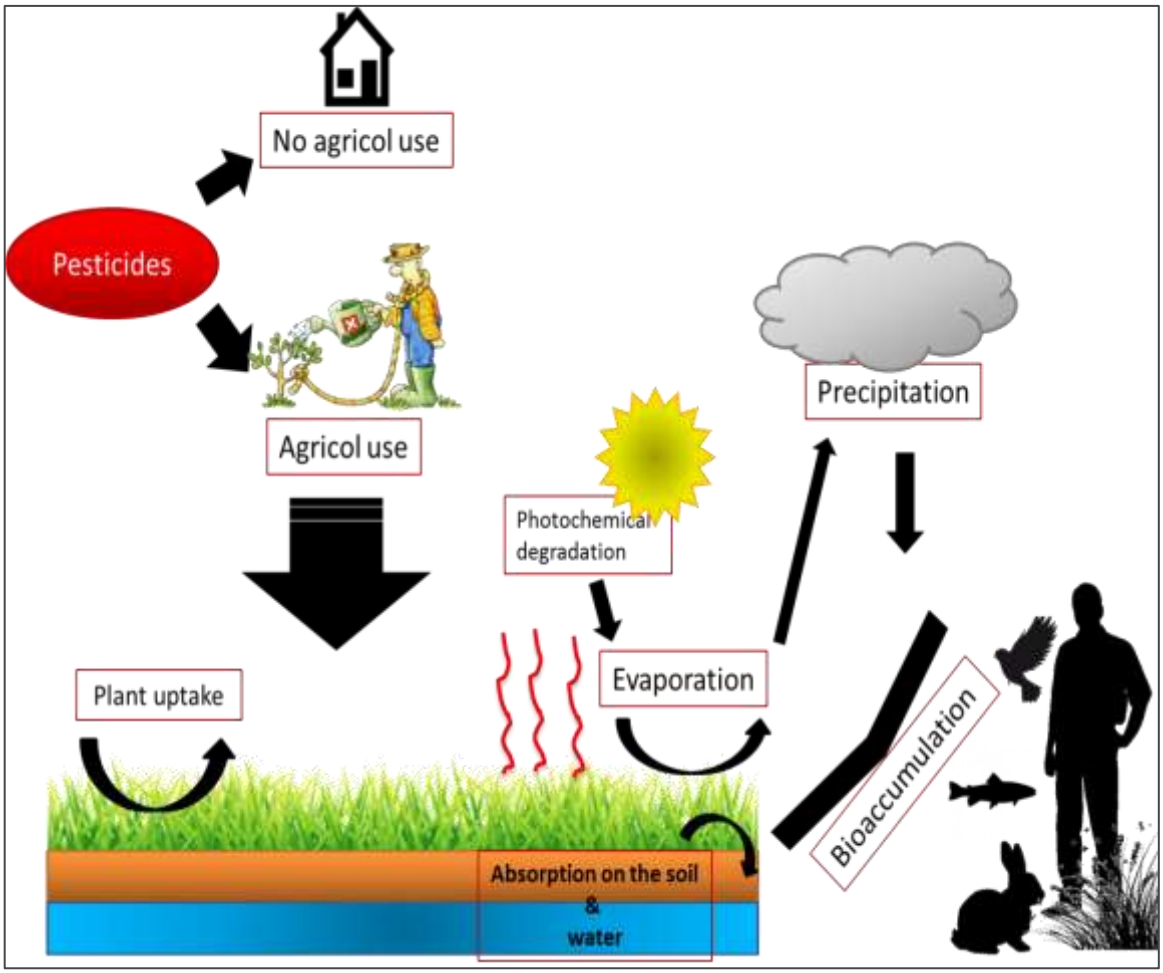


Figure 2. Behavior of pesticides in the environment **(El Mrabet et al., 2008).**

1.3. Toxicokinetics of Fungicides

Fungicide exposure pathways are multiple. Whatever is the path of penetration; humans or animals are exposed to fungicides through ingestion or absorb them through the skin or the respiratory system. Fungicides are transported by blood to all organs. In general, the liver is the primary site for biotransformation and may include detoxification as well as activation reactions. They are accumulated in adipose tissue and organs containing a high content of lipid membranes especially in nervous system (brain, spinal cord and nerves), bones, liver and muscles. Then they are excreted and eliminated through expiration, sweat, bile, feces and urine (**Gupta and Aggarwal, 2012).**

1.4. Fungicides Classification

Table 01: Chemical groups of fungicides according to their generic names **(Burpee, 2006).**

CHEMICAL GROUP	GENERIC NAMES
TRIAZOLES	PROPICONAZOLE
	TRIADIMEFON
	MYCLOBUTANIL
	TRITICONAZOLE
	TETRACONAZOLE
PYRIMIDINES	FENARIMOL
STROBILURINS	FLUOXASTROBIN
	TRIFLOXYSTROBIN
	AZOXYSTROBIN
	PYRACLOSTROBIN
POLYOXINS	POLYOXIN D
BENZIMIDAZOLES	**THIOPHANATE-METHYL**
	BENOMYL
	CARBANDAZIM
DICARBOXAMIDES	IPRODIONE
	VINCLOZOLIN
PHENYLAMIDES	MEFENOXAM
CARBAMATES	PROPAMOCARB
PHOSPHONATES	FOSETYL ALUMINUM
	PHOSPHONATE
DITHIOCARBAMATES	**THIRAM**
	MANCOZEB
AROMATIC HYDROCARBONS	QUINTOZENE
	CHLORONEB
	ETHAZOLE
PEROXIDES	HYDROGEN DIOXIDE
NITRILES	CHLOROTHALONIL
PHENYLPYROLLES	FLUDIOXONIL
CYANOIMIDAZOLE	CYANOFAMID
CARBOXAMIDES	FLUTOLANIL
	BOSCALID
BIOFUNGICIDES	ECOGUARD
	SONATA
	SOILGUARD

➢ Under chemical classification of fungicides, two fungicides are chosen: Methyl-Thiophante and Thiram, the first from Benzimidazol's and the second from Dithiocarbamat class.

1.5. Methyl Thiophanate

1.5.1. Definition

Methyl Thiophanate 1, 2-Bis (3-(methoxycarbonyl)-2-thioureido) benzene) is a classic Benzimidazole Carbamate. It is a systemic broad-spectrum fungicide that has been used for many years controlling various fungal pathogens of various food crops, ornamental plants, trees and grasses. It is used also as a preseative in paint, textile, papermaking, leather industry and warehousing practices **(Giry et *al.*, 2001; Berglof et *al.*, 2002).** The LD_{50} of Methyl Thiophanate in the male rabbit by oral route is 2270 mg/kg body weight (**Tomlin, 1994).** It is well absorbed (80–85%) after oral exposure and is subsequently metabolized into many compounds within the organisms. The main metabolite is Carbendazim (Methyl 2-Benzimidazolyl Carbamate) **(Roberts et *al.*, 1998; Cardone et *al.*, 2012)**. Carbendazim is poorly catabolized and remains in tissues such as gonads, liver, adrenals, adipose tissue, skin and other organs **(WHO, 1993)** and it is a well-recognized testicular toxicant **(Yu *et al.*, 2009)**.

1.5.2. The Chemical Structure of MT

The molecular formula of Methyl Thiophanate is $C_{12}H_{14}N_4O_4S_2$ (fig 03). It has several synonyms: [4, 4 '- (o-Phénylén) bis (3-thioallophanate) of dimethyl], Metoben, Methyl topsin, Mildothane, [1, 2-Bis (Methoxycarbonylthioureido) Benzene] and Cercobin Methyl **(Mackay et *al.*, 2006).**

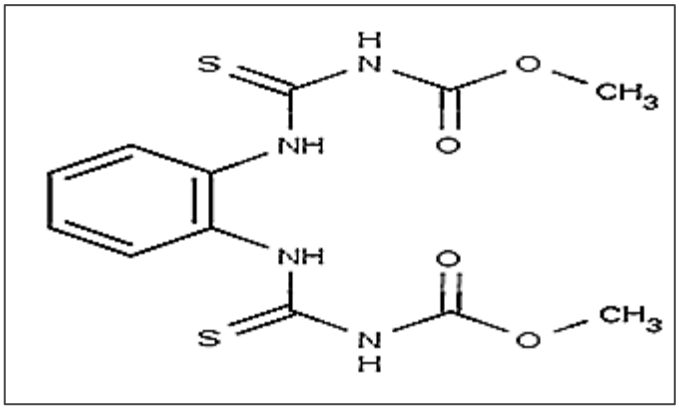


Figure 3. The chemical Structure of MT **(Mackay et *al.*, 2006)**

1.5.3. Physical and Chemical Properties of MT

Table 02: presents some physical and chemical properties of MT.

	Properties	**References**
CAS Registry No	23564-05-8	(EPA, 2007)
Molecular weight	342,40	(Lide, 2007)
Smell	Low sulfur smell	(EPA, 2007)
Density	1,4 -1,6	(Lide, 2007)
Solubility in water At 20°C	26,6 mg/l	(Wauchope et *al*., 1992)
Vapor Pressure At 25°C	7,13 x 10-8 mm Hg	(Tomlin, 2004)
Color and shape	Colorless, crystals	(Tomlin,1994)

1.5.4. Toxicokinetics of MT:

Both Methyl Thiophanate and Carbendazim are classified as Benzimidazole fungicides. Carbendazim is a major metabolite of MT with closely similar structural and toxicological characteristics (Methyl Thiophanate is also structurally related to benomyl).

In studies reported by **Noguchi et *al*. (1971)** and **Fujino et *al*. (1973)** the administered Methyl-Thiophanate was conjugate to Carbendazim (MBC). MT and Carbendazim are well absorbed after oral exposure (80–85%) but poorly absorbed after dermal exposure (1 or 2%) in rats, mice, dogs and hamsters.

Fujino et *al*. (1973) suggested the metabolism scheme shown in Fig (04). They reported that the major part of fecal excretion was in the form of unmetabolized Methyl-Thiophanate, while the minor parts consist of 4-hydroxy-thiophanate-methyl (4-OH-TM) and dimethyl-4.4'-O-phenylenebisallophanate. Methyl 2-benzimidazol carbamate (Carbendazim) and 5-hydroxy-MBC (5-OH-MBC) were also observed during the identification of metabolites of fecal extracts. It was, however, questioned whether these

two were actual metabolites in faeces or they were compounds produced during the analytical procedures from Methyl-Thiophanate and 4-OH-TM respectively.

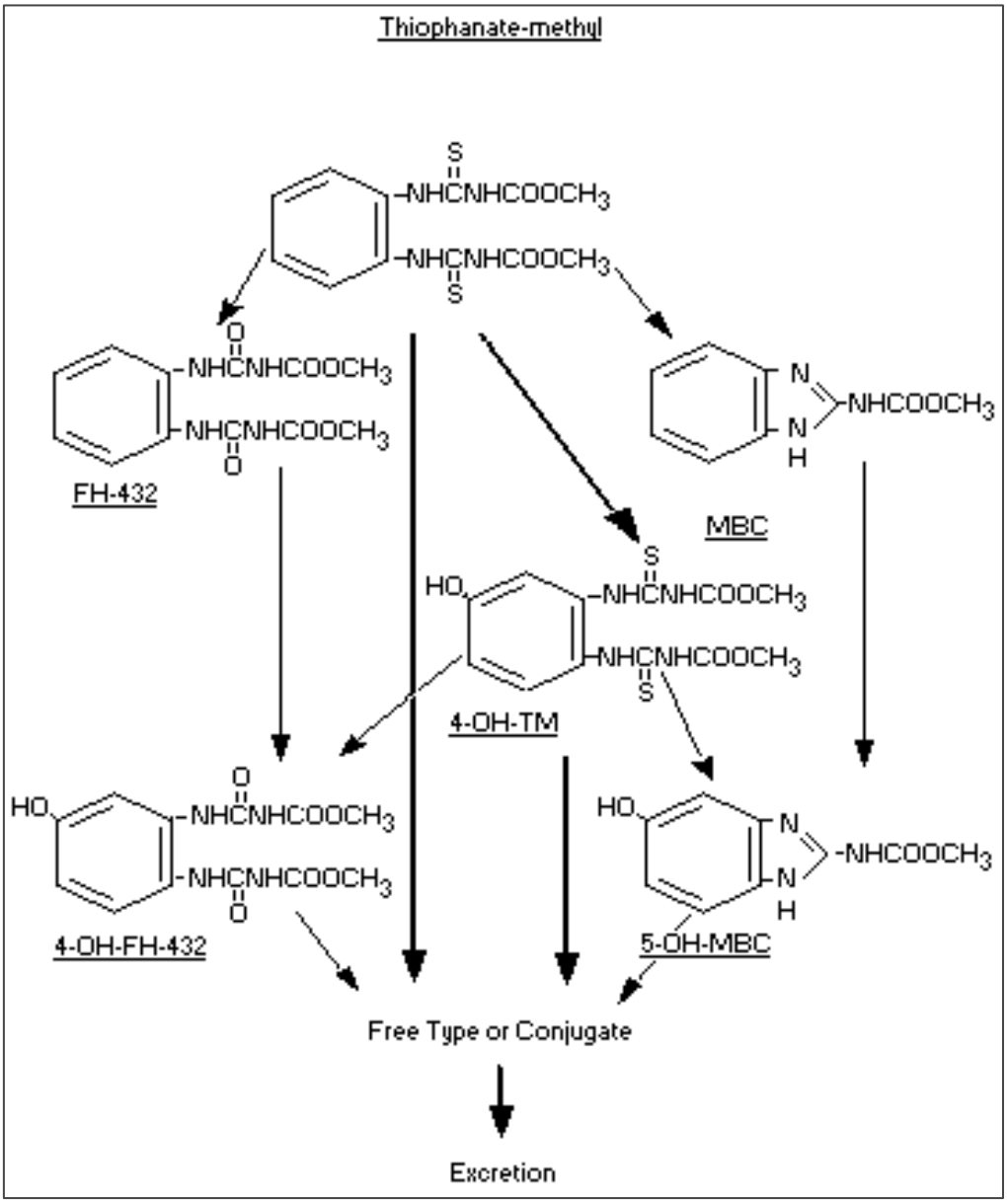


Figure 4**.** Metabolism of MT in organism **(funjo et *al.*, 1973)**

MT	:	Methyl-Thiophante
FH-432	:	Dimethyl-4.4'-o-phenylenebis (allophanate)
4-OH-FH-432	:	4-Hydroxy-dimethyl-4.4'-o-phenylenebis (allophanate)
4-OH-TM	:	4-Hydroxy-thiophanate-methyl
MBC	:	Methyl-2-benzimidazolecarbamate
5-OH-MBC	:	5-Hydroxy-methyl-2-benzimidazolecarbamate

1.5.5. Toxicodynamics of MT

Previous considerations of MT found that it has low acute oral, dermal and inhalational toxicity in animals. The main hazards associated with repeated exposure to their metabolite Carbendazim are systemic effects on liver and thymus and effects on reproduction. Furthermore Carbendazim has also been shown to produce numerical aberrations (aneuploidy) in mammalian cells exposed in vitro and in vivo **(APVMA, 2008)**.

The European Union has classified Carbendazim as a potential genotoxic chemical. Relatively information has highlighted health concerns associated with exposure to Carbendazim. Following an investigation to the effects of Carbendazim on various cell types in the testis, **Kadalmani et *al.* (2002)** observed that spermatocytes are a target for Carbendazim. **Lu et *al*. (2004)** concluded that Carbendazim causes losses of spermatozoa, decrease in testis weight and decrease in sperm concentration in rats. Carbendazim is associated with adverse reproductive effects, Furthermore both MT and Carbendazim have also been associated with an increased incidence of liver tumors and can cause aneuploidy (abnormal number of chromosomes).

The US Environmental Protection Agency has classified both Carbendazim and Methyl-Thiophanate as probable human carcinogens. Aggregate cancer risk estimates for both Carbendazim and MT from all uses including residential (lawn treatment and post-application exposure) and dietary exposure exceeded EPA's level of concern. Also it was reported that the liver and thyroid are the primary target organs of MT in several species following sub-chronic and chronic dietary exposure **(EPA, 2005)**.

1.6. Thiram

1.6.1. Definition of Thiram

Thiram (fig 05) is a Dimethyl Dithiocarbamate compound used as a fungicide to prevent crop damage in the field and to protect harvested crops from deterioration in storage or transport. Thiram is also used as a seed protectant and to protect fruit, vegetable, ornamental, and turf crops from numerous fungal diseases. Moreover, it is used as a repellent for rodents and certain large animals that cause damage to field crops. Thiram is available as dust, flowable, wettable powder, water dispersible granules, and water suspension formulations, and in mixtures with other fungicides **(Dalvi et *al*., 1988)**.

Thiram has been used in the treatment of human scabies, as a sunscreen, and as a bactericide applied directly to the skin or incorporated into soap. It is also used as a rubber accelerator in tire industry and as a lubricating oil additive **(EPA, 2004)**. The LD_{50} of Thiram in the male rabbit by oral route is 210 mg/kg body weight **(Edwards, 1991)**.

1.6.2. Chemical Structure of Thiram

The molecular formula of Thiram is $C_6H_{12}N_2S_4$. It has several synonyms (Thiram, TMTD, tetraalkyl-thiuram disulfide, bis (N,N-diméthylthiocarbamyle) disulfide **(Mackay et al., 2006)**.

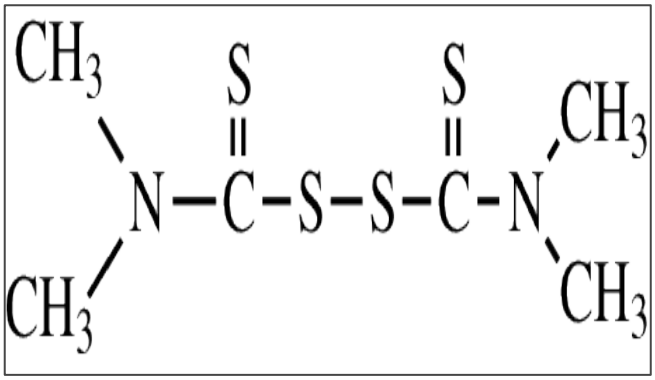


Figure 5**.** Chemical structure of Thiram **(Mackay et al., 2006).**

1.6.3. Physical and Chemical Properties of Thiram

Table 03: presents some physical and chemical properties of Thiram.

	Properties	References
CAS Registry No	137-26-8	(EPA, 2004)
		(EPA, 2004)
Molecular weight	240,4	(Lide, 2007)
		(EPA, 2004)
Smell	specific odor	(EPA, 2004)
Density	1,36 g/cm3 à 20 ° C	(Lide, 2007)
Solubility in water at 20°c	Soluble	(EPA, 2004)
Vapor pressure at 25°C	1.72x10-5 mm Hg	(Tomlin, 2003)
Color and shape	White or yellow, crystals	(Lewis, 1997)

1.6.4. Toxicokinetics of Thiram:

After absorption (respiratory, dermal, and gastrointestinal), Thiram appears to be readily absorbed through the intestinal tract and the lungs and is quickly and widely distributed throughout the body **(ACGIH, 2001)**.

It is metabolized in the body to toxic metabolites Dimethyldithiocarbamate (This is found either as the free acid or as the *S*-glucuronide conjugates) and Carbon Disulfide **(Aldridge and Magos, 1978)**, although these compounds have been shown to inhibit hepatic microsomal enzymes **(Dalvi and Deoras, 1986).**

Gay et *al.* (1992) summarized the conclusions from a series of metabolism studies on rats with [Thiocarbonyl-^{14}C] Thiram. When rats were dosed orally with [^{14}C] Thiram much of the ^{14}C (40-60%) was eliminated as volatiles in exhaled air, 25-35% was excreted in the urine and 2-5% in the faeces. After an interval of 96 hours 2-3% of the ^{14}C remained in the tissues. Polar metabolites and conjugates (Dimethyldithiocarbamate) were identified in the

urine and other metabolites include Elemental Sulfur, Methionine, Formaldehyde **(Aldridge and Magos, 1978; Verschueren, 2001).**

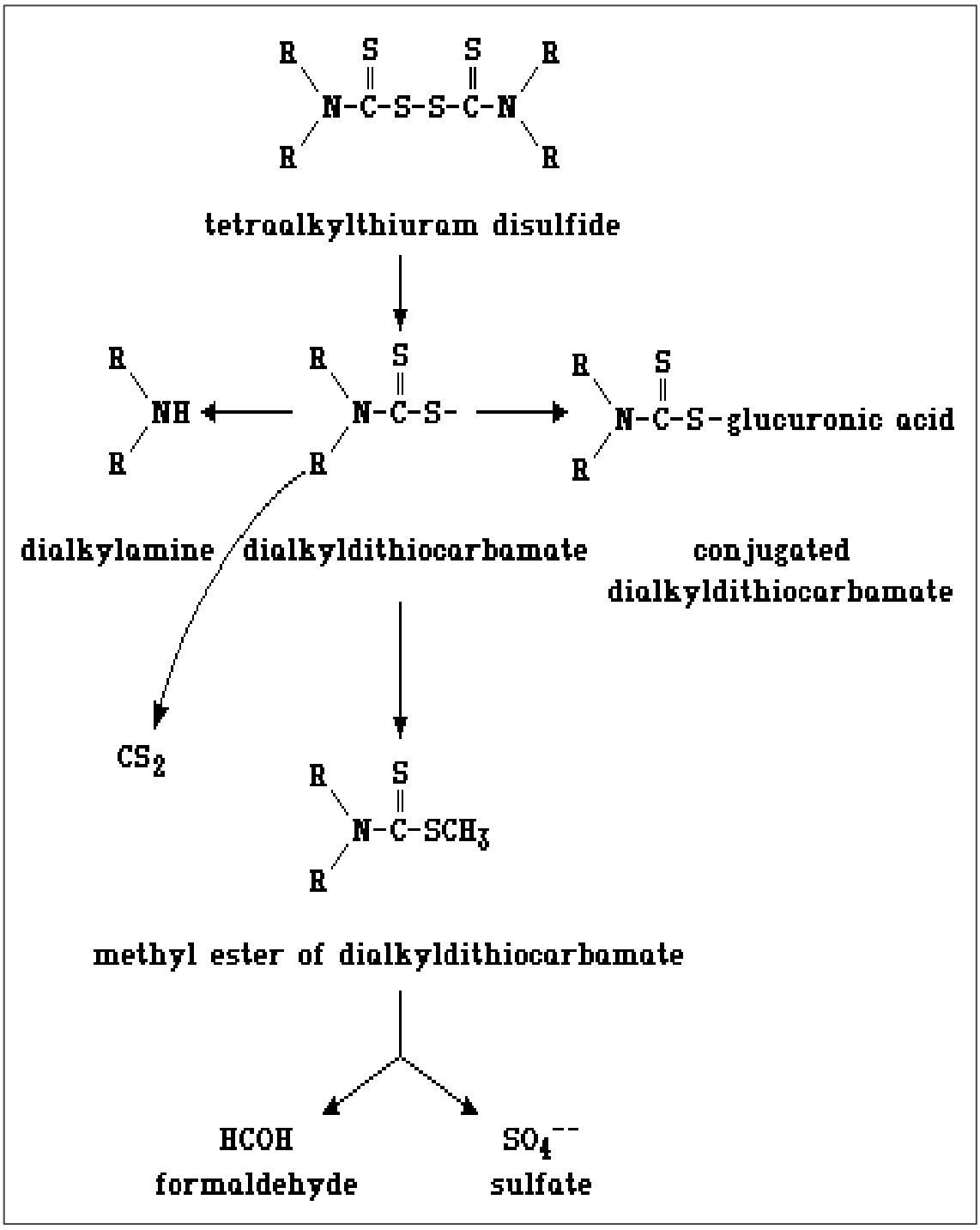


Figure 6. Metabolism of Thiram in organism **(Aldridge and Magos, 1978).**

1.6.5. Toxicodynamics of Thiram

Thiram is moderately toxic by ingestion, but it is highly toxic if inhaled. Reported oral LD50 values for Thiram are 620 to over 1900 mg/kg in rats; 1500 to 2000 mg/kg in mice and 210 mg/kg in rabbits (**Edwards et *al*., 1991; Kidd and James, 1991**). Acute exposure in humans may cause headaches, dizziness, tiredness, nausea, diarrhea, and other gastrointestinal complaints **(U.S. National Library of Medicine, 1995).**

The subchronic toxicity profile for Thiram indicates that hematology, clinical chemistry and body weight are affected after subchronic exposure to the compound for all species evaluated **(EPA, 2004).**

The chronic toxicity profile for Thiram indicates that the liver, blood and urinary system are the target organs for this chemical. Studies have shown evidence of liver damage by Thiram in the form of decreased liver enzyme activity and increased liver weight. Thiram may also cause damage to the nervous system **(US National Library of Medicine, 1995).**

Symptoms of chronic exposure to Thiram in humans include drowsiness, confusion, and loss of sex drive, incoordination, slurred speech, and weakness, in addition to those due to acute exposure. Repeated or prolonged exposure to Thiram can also cause allergic reactions such as dermatitis, watery eyes, sensitivity to light, and conjunctivitis **(Edwards et *al*., 1991).**

In a combined chronic/cancer study in rats, effects included changes in hematology parameters, increased incidence of bile duct hyperplasia **(EPA, 2004).**

Dithiocarbamate pesticides such as Thiram have a marked spermicidal activity in humans. They also cause viable and non-viable gross morphological alterations of sperm **(Rice, 1964)**. In animals, Dithiocarbamate pesticides given to young and adult domestic chickens produced retarded testicular development and atrophy **(Raasul and Howell, 1974)**.

Many investigations reported that oral doses of Thiram 49 mg/kg/day to rats for 2 years produced weakness, muscle incoordination, and paralysis of the hind legs. Rats fed 52 to 67 mg/kg/day for 80 weeks exhibited hair loss, and paralysis with atrophy of the hind legs. Symptoms of muscle incoordination and paralysis from Thiram poisoning have been shown to be associated with degeneration of nerves in the lower lumbar and pelvic regions **(Edwards et *al*., 1991).** Also toxic and some tumorigenic effects have been observed

in different animal species exposed to Thiram **(Dalvi, 1988; Maita et *al*., 1991)**. numerous tests indicated that it is genotoxic **(Crebelli et *al*., 1992; Hemavathi and Rahiman, 1996**) and that it reported also effects on cartilaginous tissues in vitro and in vivo, in different animal species exposed to Dithiocarbamtes, including Thiram, **(Suzuki et *al*., 2000, 2001; Rath et *al*., 2004; Simsa et *al*., 2007).**

Materials& Methods

2. MATERIALS AND METHODS

2.1. Fungicides

Two fungicides have been used: ***Methyl Thiophanat*** (70%) as a wettable powder and ***Thiram*** (85%) as a wettable powder, which were obtained from a pharmaceutical company.

2.2. Animals

We used mature male rabbits *Cuniculus lepus* aged between 6 and 8 months with body wight 1500 ± 500 g. The study was carried out in the animalery of the University Badji Mokhtar-Annaba. The Animals were housed in specific cages (50× 60×53 cm^3) and were maintained in natural conditions of temperature, photoperiod and relative humidity. Animals had access *ad libitum* to water and food contains all the necessary elements (salad, carrot, cabbage and beet) the same quantity is given to all the groups of rabbits.

Rabbit Classification

- Kingdom: Animalia
- Phylum: Chordata
- Class: Mammalia
- Order: Lagomorpha
- Family: Leporida
- Species: *Cuniculus lepus*

2.3. Treatment

The experimental protocol was divided into the first and the second experimentation :

- First experimentation: animals treated with **Methyl Thiophante** (Benzimidazol).
- Second experimentation: animals treated with **Thiram** (Dithiocarbamate).

One group of rabbits served as a control group was common in both experimentations.

2.3.1. The First Experimentation

The rabbits were divided into four groups (6 rabbits in each group).

- ✓ G1: Group served as a control one.

And three groups were treated orally with **Methyl Thiophanate** dissolved in water at doses:

- ✓ G2: Group treated with 50 mg/1ml / kg body weight.
- ✓ G3: Group treated with 100 mg/1ml / kg body weight.
- ✓ G4: Group treated with 150 mg/1ml / kg body weight.

MT was administered by oral system for 4 weeks.

2.3.2. The Second Experimentation

The rabbits were also divided into four groups, each one contained 6 rabbits

- ✓ G1: Group 01 served as a control group.

Three groups were treated orally with **Thiram** dissolved in water at doses:

- ✓ G2: Group treated with 20 mg/1ml / kg body weight.
- ✓ G3: Group treated with 30 mg/1ml / kg body weight.
- ✓ G4: Group treated with 80 mg/1ml / kg body weight.

Thiram was administered by oral system over the period of 2 weeks.

- After sacrifice by decapitation, blood and semen were collected. Then, the organs of each animal of the different groups were removed and weighed.

2.4. Semen Sampling

To estimate the effects of the used fungicides on fertility of rabbits through the characteristics of spermatozoa and semen quality (count, motility, speed and viability). We proceeded to the sperm test using the method of **WHO (1993)**.

Immediately after dicapitation, semen was collected from a small opening made at the head of the epididymis. 1 µL of sperm was diluted in 49 µL of NaCl 0.9% and placed in an oven at a temperature of 37° C.

2.5. Blood Collection

Blood is immediately collected in three types of tubes, Haparin tubes, dry tubes, and the others contain the EDTA anticoagulant:

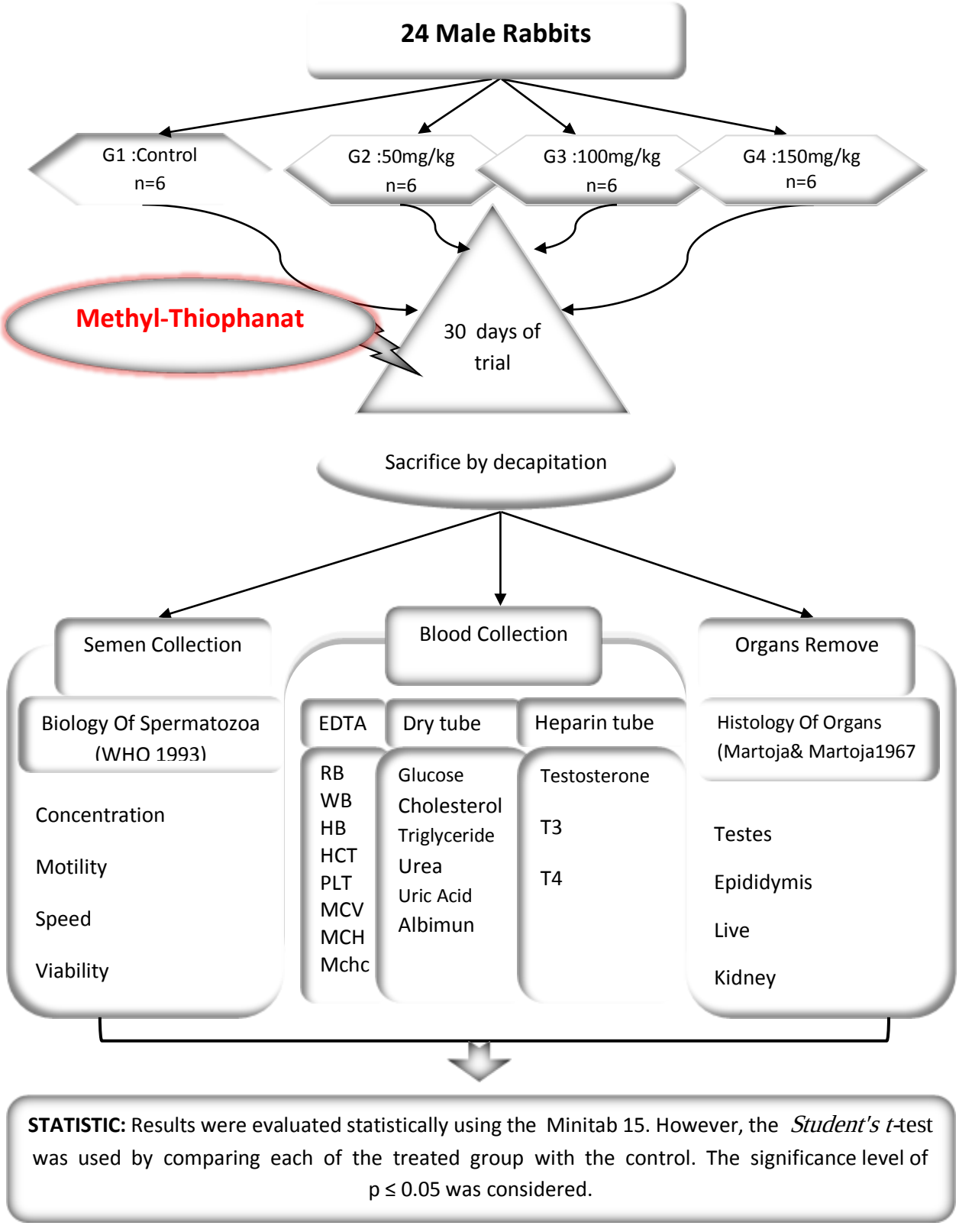
- The Tubes with EDTA: used for the determination of the formula of blood numeration.
- The Dry Tubes: centrifuged with 5000 tours/minute during 15 minutes; the recovered serums used for the determination of the biochemical parameters (Glucose, Cholesterol, Triglyceride, Uric Acid, Urea and Albumin).
- The Heparin Tubes : containing Heparin anticoagulant, the tubes are centrifuged with 5000 tours/minute during 15 minutes; the recovered plasma used for the determination of hormons (Testosteron, T3 and T4).

2.6. Organs Remove

We made a longitudinal abdominal opening in animals for sampling the different organs (testes, epididym, liver and kidneys). After the separation of their adipose tissues, the organs are weighed, then a piece of liver, kidney, epididymis and testes of each animal are fixed in formol (10%) to make histological sections.

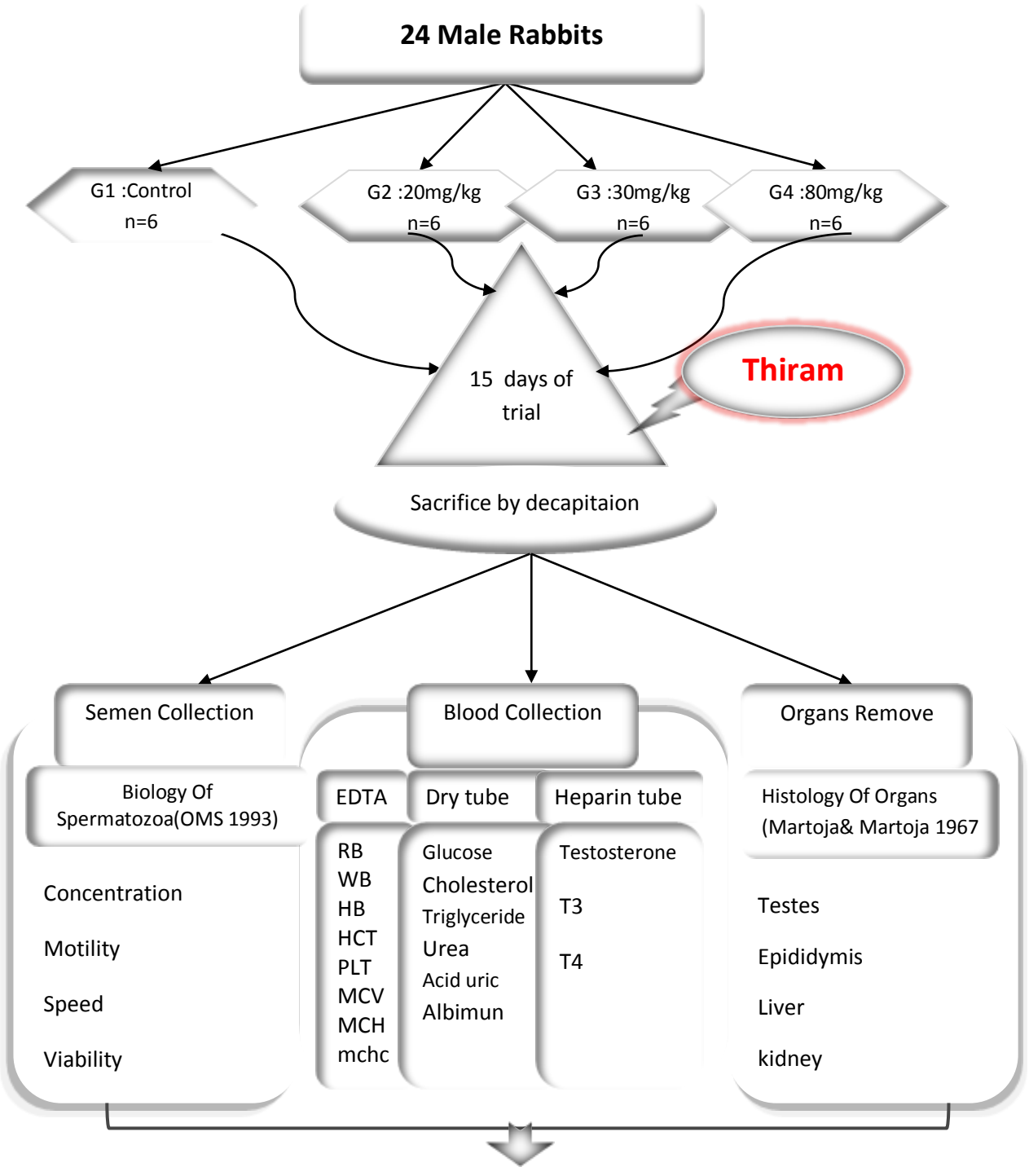
2.7. Experimental Protocol

- The next figure summarizes the various steps of the experimental protocol of the first experimentation using MT.



STATISTIC: Results were evaluated statistically using the Minitab 15. However, the *Student's t*-test was used by comparing each of the treated group with the control. The significance level of $p \leq 0.05$ was considered.

- The next figure summarizes the various steps of the experimental protocol of the second treatment using Thiram.



STATISTIC: Results were evaluated statistically using the Minitab 15. However, the *Student's t*-test was used by comparing each treated group with the control. The significance level of $p \leq 0.05$ was considered.

2.8. Methods

2.8.1. Semen Analysis

Semen analysis is a test that seeks to assess the following characteristics of spermatozoa: (concentration, motility, speed and viability).

2.8.1.1. sperm count

Spermtozoa count was measured using a Malassez cell **(WHO, 1993).** After mixing the diluted semen sample, we introduce one drop into the Malassez cell then covered with a cover-slip. This study is based on counting the spermatozoa in 05 squares at magnification (× 40) under the microscope. The sperm count calculated via the following method:

$$\text{Concentration } (x10^6 / \text{ml}) = \frac{D \cdot V \cdot n}{N}$$

D: Dilution ratio (50)

V: Volume of the Malassez cell

n: The number of spermatozoa counted in 05 squares

N: The number of small squares.

2.8.1.2. Motility of Spermatozoa

A drop of semen was put between normal slide and cover slip, then examined under optical microscope with magnification (× 40). Sperm motility is determined by the count of mobile spermatozoa in 3 observation fields. Then the average percentage of mobile spermatozoa is calculated **(WHO, 1993).**

2.8.1.3. Speed of Spermatozoa

First,one drop of the diluted semen was placed on the Malassez slide using a micropipette and covered with a cover slip, then the sample under microscope was examined with magnification (× 40) **(WHO, 1993).**

Finally, we evaluated the speed of sepermatozoas by counting the time (in seconds) required to cross distance of 0.05 mm between two parallel lines of 10 spermatozoas using a timer. Then the speed was calculated by applying the following formula:

$$\text{Speed } (\mu m/s) = \frac{\text{Distance } (\mu m)}{\text{Time (s)}}$$

- We calculate the speed of 10 spermatozoas, then the average speed.

2.8.1.4.Viability of Spermatozoa

❖ **Vital stain:** This is a staining technique based on the principle that dead sperm have multiple holes in their membranes, which means they will take up eosin and appear pinkish in colour. Normal live sperm will not be stained as they have intact membranes.

- The used reactive: Eosin (0.1%).

A drop of sperm + drop of eosin were put between normal slide and cover slip and let dry for 2 to 3 min.

After performing the stain test, the preparation is examined microscopically and 100 spermatozoa are recorded. Then, how many of these 100 are stained as well as how many have repeled the staining. This will allow us to count the percentage of dead and normal live sperm **(WHO, 1993).**

❖ **Hypo-osmotic swelling test:** This test is carried out to study the viability by studying the morphological changes in spermatozoa tail.

Preparation of the used Solution: Dissolve 0.367g of sodium citrate ($Na_3C_6H_3O_7$; $2H_2O$) and 0.675g of fructose in 50 ml of distilled water. After thawing, the solution is mixed well before using it.

procedure of work: we put 1 ml of the used solution in Eppendorf tube and placed it in a water bath at a temperature of 37 °C for 3 minutes. Then 0.1 ml of semen is added to the previous solution then incubate at 37 °C for 30 minutes.

After microscopic observation with magnification [40x], the percentage of spermatozoa with abnormalities was counted (fig 07) on a total counted spermatozoa **(WHO, 1993).**

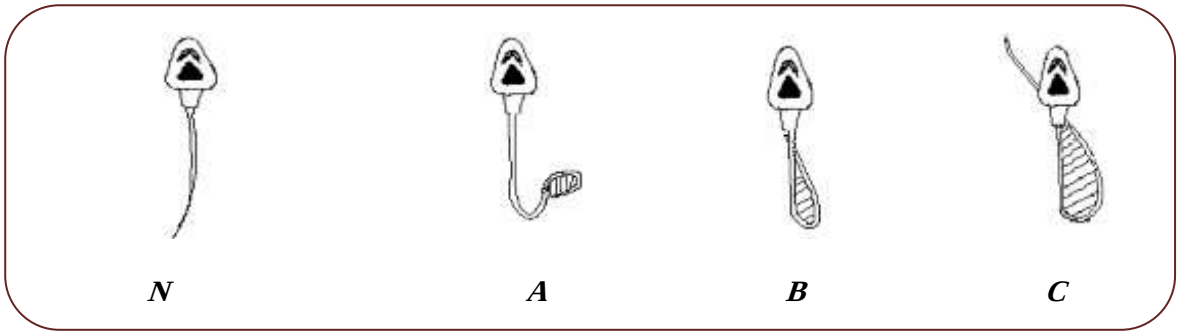


Figure 7**.** Morphological malformation of spermatozoa tail exposed to hypo- osmotic stress.

N: No malformation in the tail

A: Low malformation of the tail

B: Major malformation at the tail

C: Very important malformation in the tail and the midpiece part.

2.8.2. Hematological Analysis (Blood Count)

After decapitation, blood was collected in tubes containing EDTA anticoagulant, and was used for counting and sizing leukocytes subpopulations using automatic Coulter counter machine to calculate each of red blood cells (RBC), haemoglobin (HGB), hematocrite (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT) and total white blood cell count (WBC).

2.8.3. Hormons Assay

After collecting blood, samples were centrifuged at 5000 tr/min to isolate plasma used for mesearunig hormons. The hormones are proportioned by the conventional method ELISA using commercial kit. Measurement is done using "Tecan ELISA reader" provided with data processing software which calculates the range standard automatically and the level of the testosterone, T3 and T4 to the used unit directly.

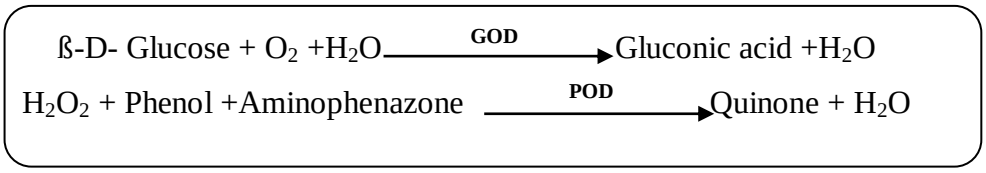
2.8.4. Biochimical Assay

We used Spinreact kit.

2.8.4.1.Quantitative Determination of Glucose

Principle of The Method

Glucose Oxidase (GOD) catalyses the oxidation of Glucose to Gluconic acid. The formed Hydrogen Peroxide (H_2O_2) is detected by a chromogenic Oxygen acceptor, Phenol-aminophenazone in the presence of Peroxidase (POD):



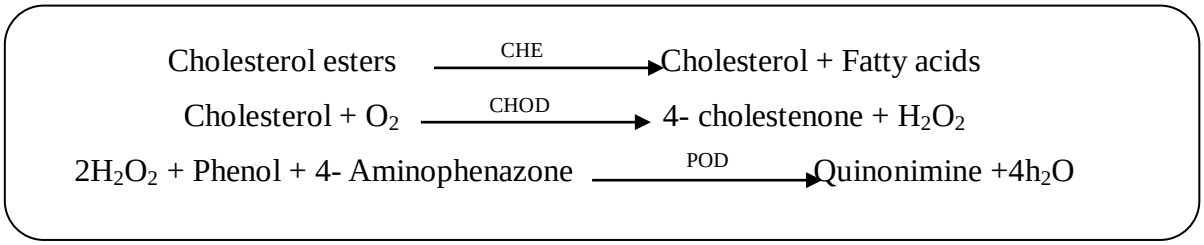
The intensity of the color formed is proportional to the glucose concentration in the sample

	Blank	Standard	Sample
WR (ml)	1,0	1,0	1,0
Standard (µl)	--	10	--
Sample (µl)	--	--	10

2.8.4.2.Quantitative Determination of Cholesterol

Principle of the Method

The cholesterol present in the sample originates a coloured complex, according to the following reaction:



The intensity of the color formed is proportional to the cholesterol concentration in the sample **(Naito, 1984)** .

	Blank	Standard	Sample
WR (mL)	1.0	1.0	1.0
Standard (µl)	--	10	--
Sample (µl)	--	--	10

2.8.4.3. Quantitative Determination of Triglycerides

- **Principle of the Method**

Sample Triglycerides incubated with Lipoproteinlipase (LPL), liberate Glycerol and free fatty acids. Glycerol is converted to glycerol-3-phosphate (G3P) and Adenosine-5-Diphosphate (ADP) by Glycerol Kinase and ATP. Glycerol-3-phosphate (G3P) is then converted by Glycerol Phosphate Dehydrogenase (GPO) to Dihydroxyacetone Phosphate (DAP) and Hydrogen Peroxide (H_2O_2).

In the last reaction, Hydrogen Peroxide (H_2O_2) reacts with 4-aminophenazone (4-AP) and p-chlorophenol in the presence of Peroxidase (POD) to give a red colored dye

$$\text{Triglycerides} + H_2O \xrightarrow{\text{LPL}} \text{Glycerol} + \text{free fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerolkinase}} \text{G3P} + \text{ADP}$$

$$\text{G3P} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AP} + \text{p-Chlorophenol} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

The intensity of the color formed is proportional to the Triglycerides concentration in the sample **(Buccolo et *al.*, 1973).**

	Blank	**Standard**	**Sample**
WR (mL)	1.0	1.0	1.0
Standard (µl)	--	10	--
Sample (µl)	--	--	10

2.8.4.4. Quantitative Determination of Uric Acid

- **Principle of the Method**

Uric acid is oxidized by Uricase to Allantoine and Hydrogen Peroxide ($2H_2O_2$), which under the influence of POD, 4–aminophenazone (4-AP) and 2-4 Dichlorophenol Sulfonate (DCPS) forms a red Quinoneimine compound:

$$\text{Uric acid} + 2H_2O + O2 \xrightarrow{\text{Uricase}} \text{Allantoine} + CO2 + 2H_2O_2$$

$$2H_2O_2 + \text{4-AP} + \text{DCPS} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4H_2O$$

The intensity of the red color formed is proportional to the Uric acid concentration in the sample (**Schultz, 1984).**

	Blank	Standard	Sample
WR(ml)	1.0	1.0	1.0
Standard (µl)	--	25	--
Sample (µl)	--	--	25

2.8.4.5. Quantitative Determination of Urea

- **Principle of the Method**

Urea in the sample is hydrolized enzymatically into Ammonia (NH_3) and Carbon Dioxide (CO_2).

Ammonia ions formed react with α-ketoglutarate in a reaction catalysed by Glutamate Dehydrogenase (GLDH) with simultaneous oxidation of NADH to NAD^+:

$$\text{Urea} + H_2O + 2\,H^+ \xrightarrow{\text{Urease}} 2\,NH_3 + CO_2$$

$$2\,NH_3 + \alpha\text{- Ketoglutarate} + \text{NADH} \xrightarrow{\text{GLDH}} H_2O + NAD^+ + \text{L-Glutamate}$$

The decrease in concentration of NADH is proportional to urea concentration in the sample (**Kaplan, 1984).**

	Blank	Standard	Sample
WR (mL)	1.0	1.0	1.0
Standard (µL)	--	10	--
Sample (µL)	--	--	10

2.8.4.6. Quantitative Determination of Albumin

- **Principle of the Method**

Albumin in the presence of Bromcresol green at a slightly acid pH, produces a colour change of the indicator from yellow-green to green-blue. The intensity of the color formed is proportional to the Albumin concentration in the sample (**Gendler, 1984).**

	Blank	Standard	Sample
R (mL)	1.0	1.0	1.0
Standard (µL)	--	--	--
Sample (µL)	--	--	5

2.8.5. Histology Examination

Histological sections were performed in anatomy laboratory at Ibn Rushd Hospital - Annaba. The technique was conducted according to the method of **Martoja and Martoja (1967)**, which comprises the following steps:

- **Fixation**

The primary function of a fixative is to preserve the cellular structure of the tissue. Fixation is necessary to protect and harden the tissue against the deleterious effects of later procedures, which, otherwise, would disrupt cellular structure beyond recognition. Furthermore, fixation minimizes a process called autolysis. Autolysis is the degradation of the cellular structure which results from the release of degradative enzymes from the excised tissue itself. The fixation process must be started as quickly as possible after removal of the sample. We let the organs section stand in the used fixative (Formol 10%) for 48 hours over the cuts.

- **Dehydration**

As the paraffin is not miscible with water, the samples must then be completely dehydrated prior to paraffin embedding. This latter is not soluble in the alcohol used for dehydration, thus, there is a substitution with xylene. Dehydration is done through a machine making immersing samples in baths of ethanol at increasing concentration (70, 95 and 100%) and in baths of xylene which is a lightening agent giving the tissue more transparency. Then,

an oven of xylene evaporates the anatomical pieces; this step is performed by a machine called the circulator.

- **Inclusion and Achievement of Blocks**

The parts are immersed in molten liquid paraffin baths at 60 ° C. The samples are soaked in paraffin and placed in molds, and then it filled with paraffin. This operation uses devices called “inclusion” Chilled to achieve rapid solidification (10 to 15 min) of the paraffin block containing the pieces of the organs.

- **Making Cuts and Staining**

- ✓ The inclusion blocks are glued on a sample holder.
- ✓ The bonding surface is softened using a heated metal blade moderately flame then placed on the object carrier block on which the block adheres very well after a slight pressure.
- ✓ The serial sections are made using a microtome
- ✓ These series of sections are connected together in strip form, which facilitates reconstitution (three-dimensional) of the observed structures. The slice thickness is 4 to 7 microns.
- ✓ The obtained tape sections are placed on glass slides
- ✓ Then covered with distilled water and placed on a hot plate (the temperature of which is lower than the melting temperature of the paraffin).
- ✓ The heat allows the staggering cuts ribbons.
- ✓ The Melting paraffin sections were stained with Hematoxylin and Eosin (H&E stain).
- ✓ Dried preparation in alcohol baths and mounted in Canada Balsam.
- ✓ Finally, the Observation of sections is performed using a light microscope.

2.8.6. Statistical Analysis

All results were expressed as Mean ± Standard Deviation (M ± SD) and analyzed using *Student's t-test* with the Minitab program (version15) comparing each treated group with the control. The significance level of $P \leq 0.05$ was considered.

Differences are considered :

- ✓ Significant (* $P \leq 0.05$).
- ✓ Highly significant compared with the control (** $P \leq 0.01$).
- ✓ Very highly significant compared with the control (*** $P \leq 0.001$).

Results

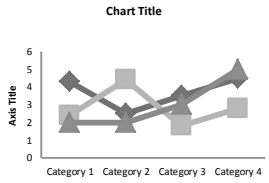


Chart Title
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6
5
4
3
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Category 1
Category 2
Category 3
Category 4

3. RESULTS

3.1. Impact of Methyl Thiophanate During the First Experimental Protocol

3.1.1. Experimental Observation of the Effects of MT

3.1.1.1. Variation of Organs Weight

The statistical analysis of these results revealed a significant reduction ($p \leq 0.05$) in weight of kidney in the group treated with the high dose of MT. It was also significantly decreased ($p \leq 0.01$, $p \leq 0.001$) in weight of testis and epididymis in all the treated groups compared with the control group.

However we noted a significant increase ($p \leq 0.05$, $p \leq 0.01$) of liver weight in the treated groups G3 and G4 compared with the control group.

Table 04: Organs weights of experimental rabbit treated with MT compared with the control group (n=6).

Weight (g)	G1: Control	G2: 50mg/kg	G3: 100mg/kg	G4: 150mg/kg
Liver	54.85±3.54	59.15±2.66	60.49±4.23*	67.16±5.2**
kidney	5.65±0.70	5.42±0.26	5.38±0.17	5.28±0.08*
Testes	2.27± 0.20	1.22± 0.19***	0.99± 0.20***	1.09 ±0.07***
Epididymis	0.50± 0.11	0.28± 0.06**	0.23± 0.08**	0.22 ±0.05***

$\bar{x} \pm$ SD (n=6) P≤0.05*, P≤0. 01**, P≤0.001***

3.1.1.2. Macroscopic Observation of liver

The Photographs of control rabbit exhibited a normal appearance of liver (fig 8,a). In the contrast the macroscopic observation (fig 8,b,c,d) of liver of the treated groups with MT illustrating a gross morphological changes revealed by fat accumulation, with presence of irregular nodules emerged on the liver surfaces.

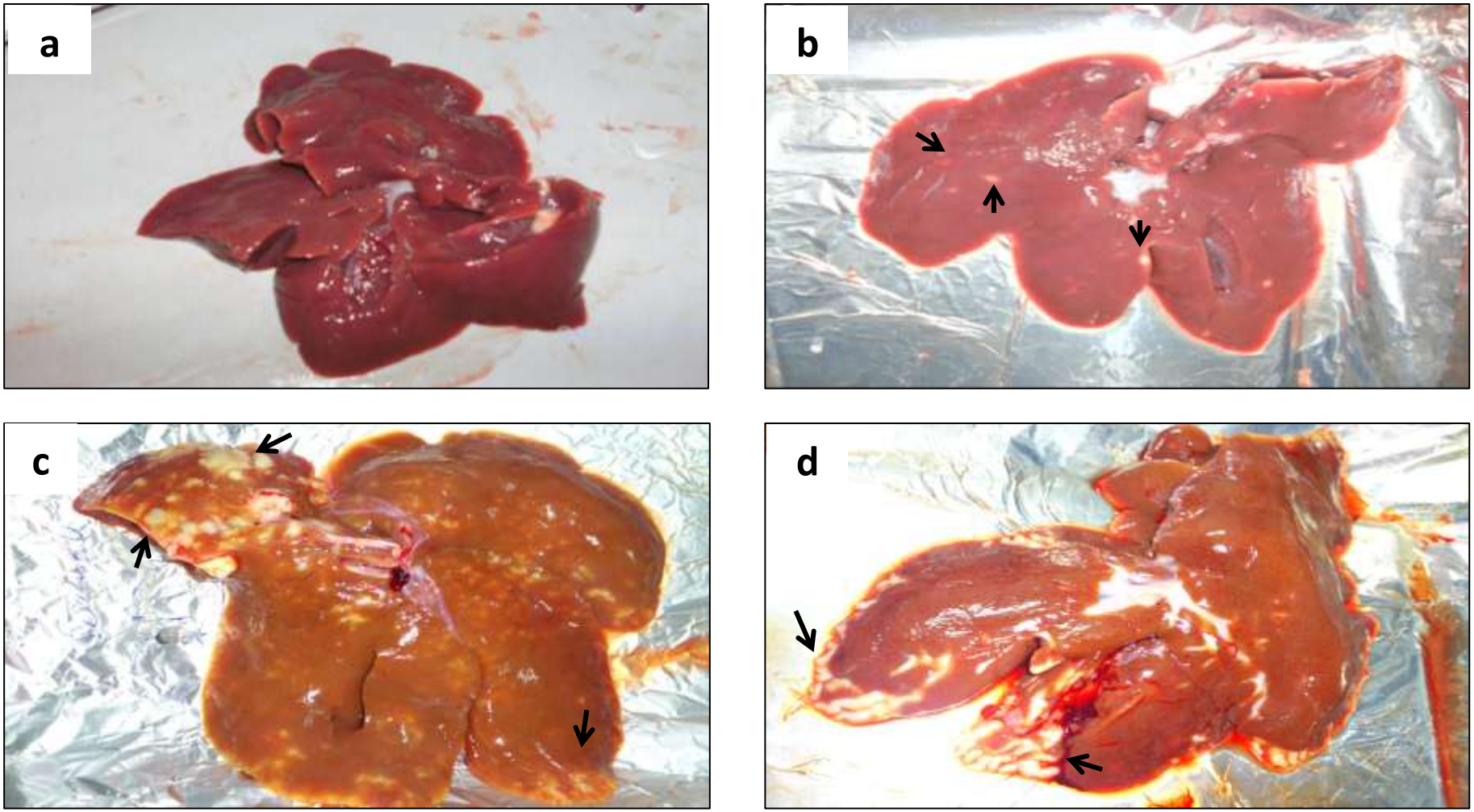

Figure 8. Photographs illustrating the gross morphological changes of liver of the treated rabbits with MT and the control rabbit/ **a:** normal liver of control rabbit, **b:** Treated rabbit with 50mg/kg of MT, **c:** Treated rabbit with 100 mg/kg of MT, **d:**Treated rabbit with 150 mg/kg of MT. **Arrows:** irregular nodules (showing yellowish-white coloration and hardening) emerged on the liver surfaces **Note:** severe fatty change was observed in liver surfaces of rabbit from the treated groups with high doses of MT compared with the control group**.**

3.1.2. Impact of MT on Fertility

The Epididymal sperm analysis showes a significant decrease (P≤0.001) of sperm count (Fig 9), motility (Fig 10) and speed (fig 11) in the treated groups compared to the control group.

3.1.2.1. Sperm Concentration

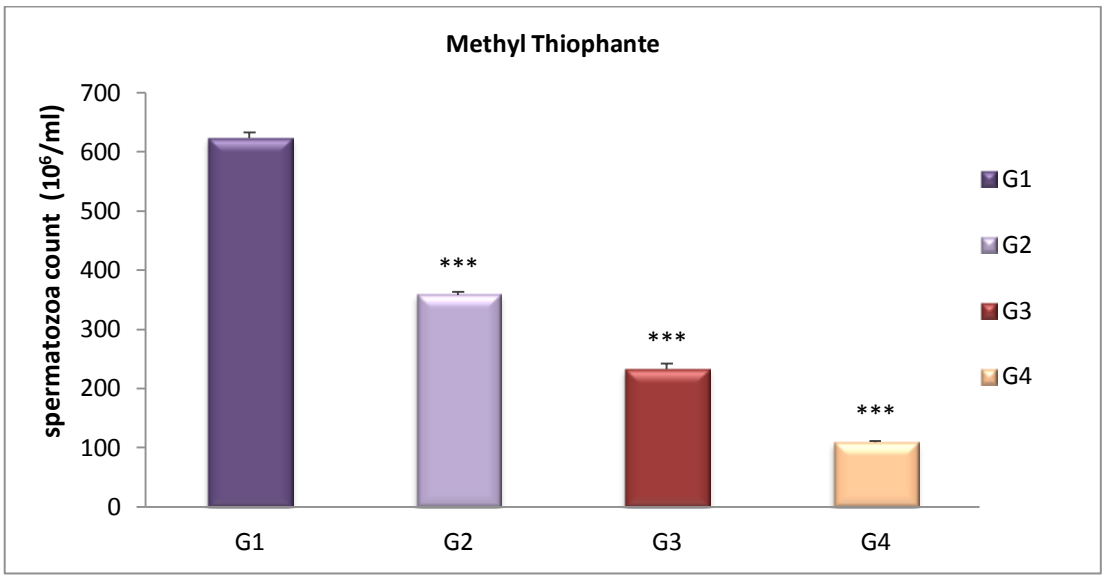

Figure 9. Epididymal sperm count of treated groups (x10^6/ml) compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.001***).

3.1.2.2. Motility of Spermatozoa

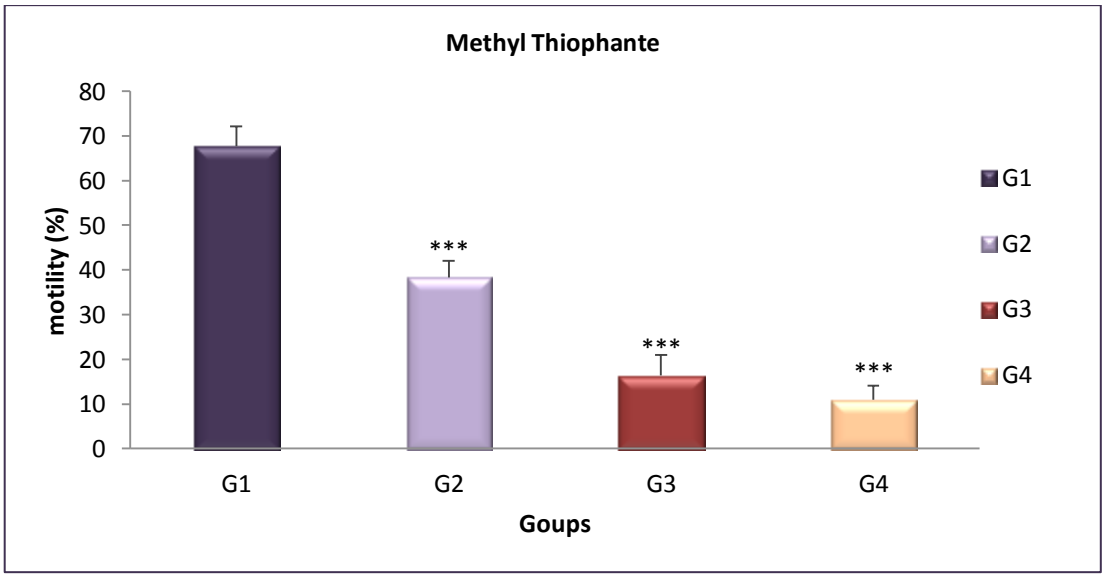

Figure 10. Epididymal sperm motility (%) of treated groups with MT compared to the control one after 4weeks ($\bar{x} \pm$ SD, n=6. P≤0.001***).

3.1.2.3. Speed of Spermatozoa

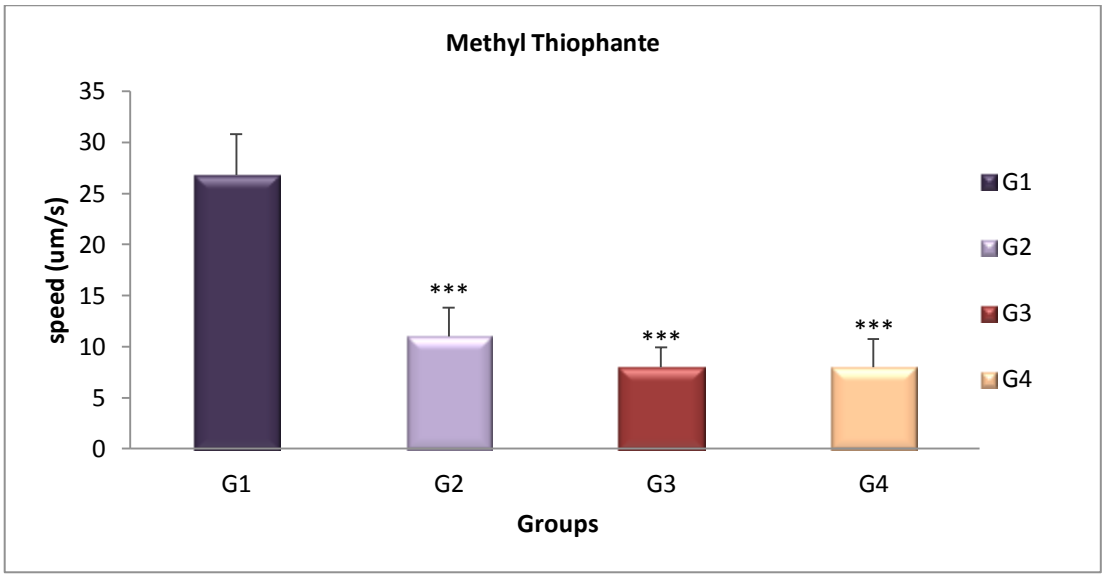

Figure 11. Epididymis sperm speed (µm/s) of treated groups with MT compared with the control group after 4 weeks ($\bar{x}$ ± SD,n=6. P≤0.001***)

3.1.2.4.Viability of Spermatozoa

- **Eosin Test**

Data present in fig (12) indicates a significant decrease in the percentage of alive spermatozoa with an increase in the dead spermatozoa in the treated groups with MT compared with the control one.

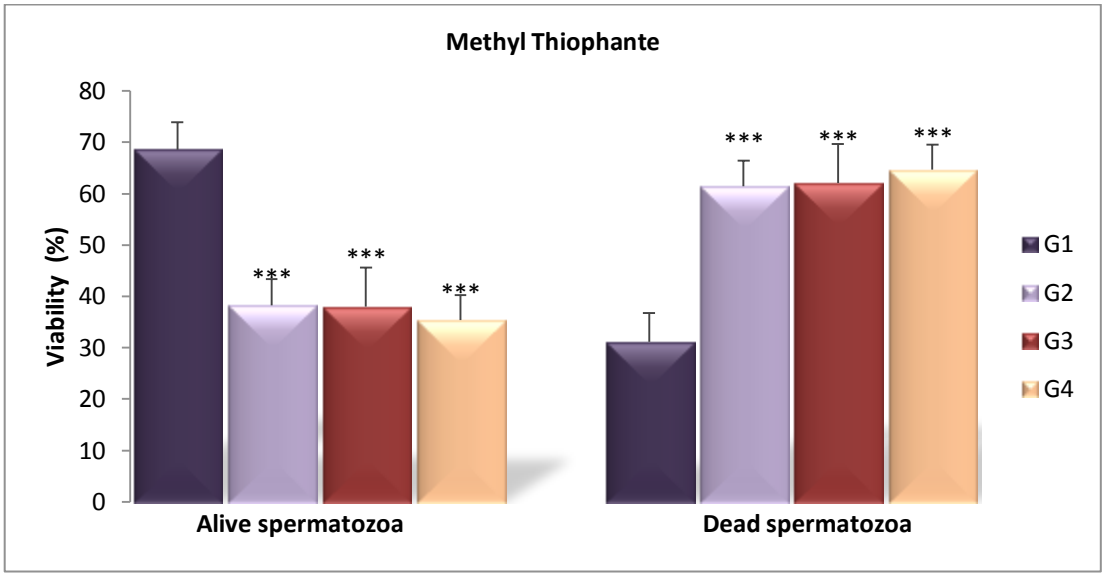

Figure 12. Epididymal sperm viability (%) of treated groups with MT compared with the control one after 4 weeks ($\bar{x}$ ± SD,n=6. P≤0.001***).

Hypo-osmotic Swelling Test

Data in Fig (13) shows a significant increase ($p \leq 0.001$) of dead spermatozoa (N) in the treated group compared with the control one.

The percentage of alive spermatozoa type (A) significantly decreased ($p \leq 0.05$, $p \leq 0.01$) in the experimental groups compared to the control one.

The percentage of alive spermatozoa type (B) significantly reduced ($p \leq 0.05$) in the treated groups compared with the control one.

Also, we noted a significant decrease in percentage of alive spermatozoa type (C) ($p \leq 0.01$, $p \leq 0.001$) in the treated groups compared with the control one.

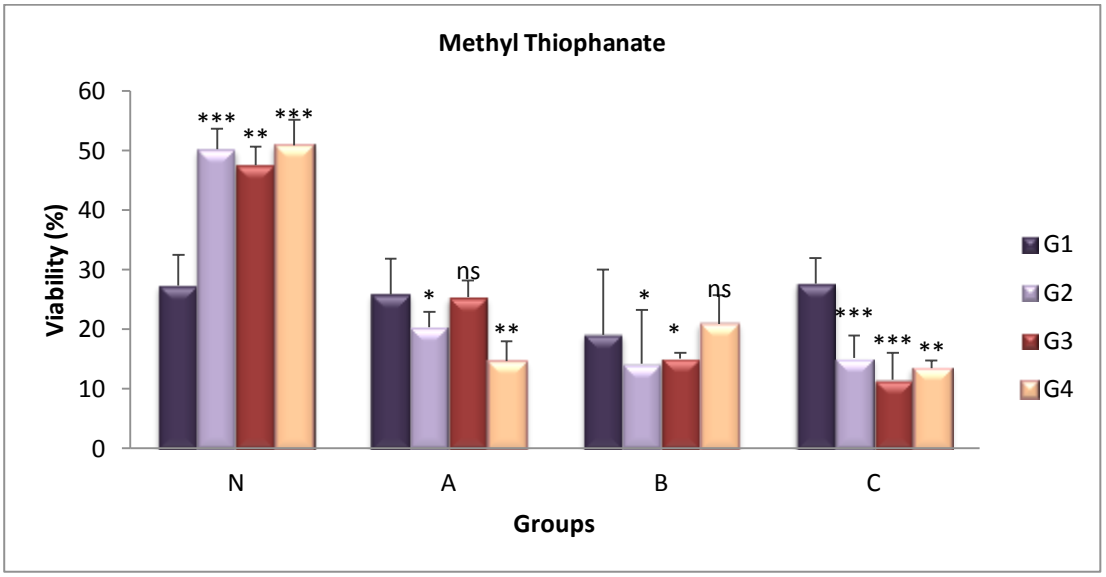

Figure 13. Epididymal sperm viability (%) of treated groups with MT compared with the control one after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*, P≤0.01**, P≤0.001***)

3.1.2.5. Plasma Testosterone Level

The changes in Testosterone level in all groups of rabbits is reported in the Fig 14. The results revealed that the plasma Testosterone levels significantly decreased ($P \leq 0.01$, $p \leq 0.001$) in MT treated rabbits compared with the control animals.

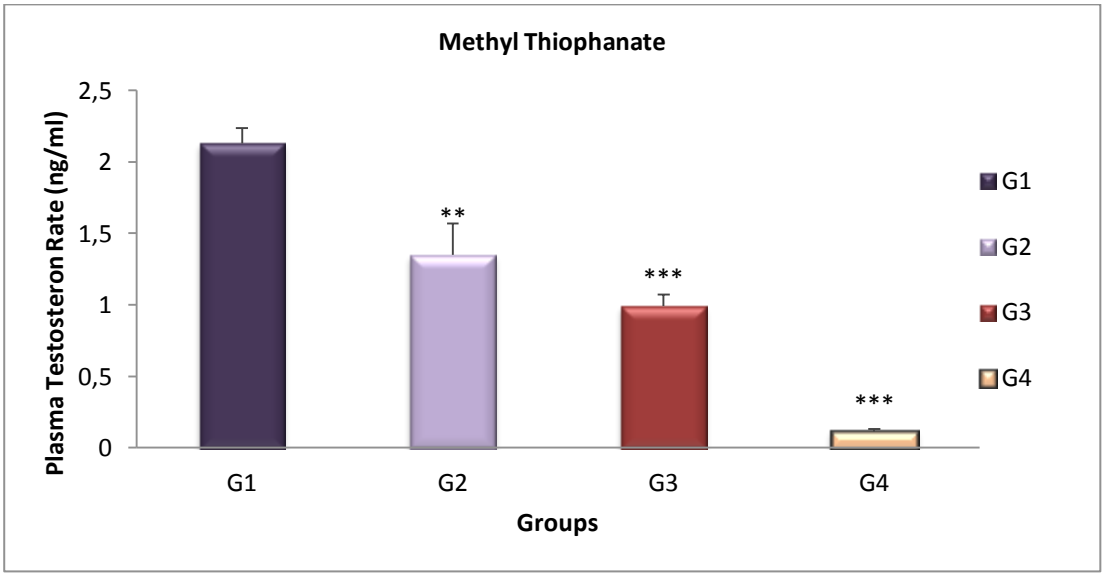

Figure 14. Variation of Testosterone level (ng/ml) of treated groups compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**, P≤0.001***).

3.1.3. Impact of MT on Hematological Parameters

Red blood cells, hemoglobin and hematocrit significantly decreased in the group treated with the high dose of MT compared with the control group.

White blood cells increased in the group treated with 50 mg/kg, while decreased significantly (p≤0.01) in the groups treated with 100 and 150 mg/kg compared with the control group.

Platelets significantly decreased (p≤0.01, p≤0.001) in the groups treated with 100 and 150 mg/kg compared to control group whereas the MCV, MCH and MCHC were not significantly affected at all the treated groups.

Table 05: variation of hematological parameters of treated groups with MT compared with the control group after 4 weeks (n=6).

Parameters	G1/ control	G2/ 50mg/kg	G3/ 100mg/kg	G4/ 150 mg/kg
RBC (10^{12}/l)	6.15±0.81	5.53±0.17	5.38±0.23	4.68±0.25*
WBC (10^{9}/l)	6.89±1.22	6.60±1.4	2.5±1.12**	2.43±1.54**
HGB (g/dl)	115±8.74	107.6±14.7	102.23±8.08	84.5±7.41**
HCT (%)	35.3± 3.1	35.23± 8.8	31.2± 6.08	27.58± 1.47 *
PLT	261±19.3	245.67±9.29	112±23.6**	104.3±18.4***
MCV	58.75±3.59	56.25±1.26	52±6.08	51.25±5.38
MCH	15.75±3.7	17.48±2.79	19.57±4.28	19.17±0.86
MCHC	312.8±30.9	309.5±45.4	308±22.5	307.5±34.5

($\bar{x} \pm$ SD, P≤0.05*, P≤0.01**, P≤0.001***)

3.1.4. Impact of MT on Thyroids Hormones

The plasma tri-iodothyronine rate (fig 15) significantly decreased (p≤0.05, p≤0.01, p≤0.001) in all the groups treated with MT compared with the control group, whereas the plasma thyroxin rate (fig 16) was not significantly affected in the group treated with the low dose of MT, while it was significantly reduced (p≤0.01) in the groups treated with 100 and 150 mg/kg of MT as compared with the control one.

3.1.4.1.Tri-iodothyronine (T3)

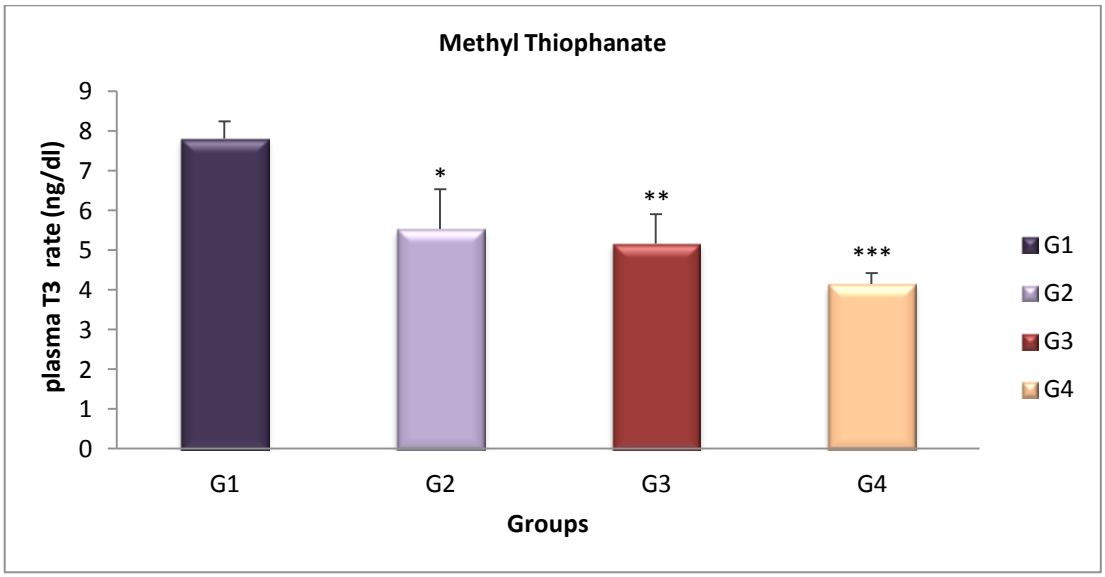

Figure 15. Variation of plasma T3 rate (ng/ml) of the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*,P≤0.01**, P≤0.001***).

3.1.4.2. Thyroxin (T4)

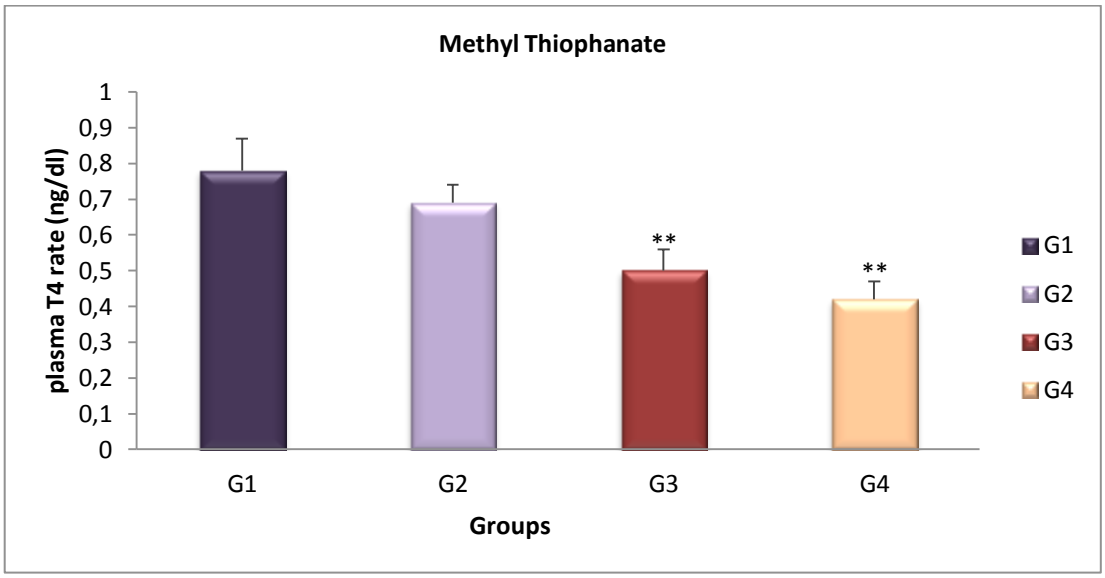

Figure 16. Variation of plasma T4 rate (ng / ml) of the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**).

3.1.5. Impact of MT on Biochemical Parameters

The results obtained in our study and shown in figure (17) reveal that the blood Glucose rate increased significantly ($p \leq 0.01$) in the treated groups G3 and G4 compared with the control one.

From figure (18), the result showed that the Cholesterol level increased significantly ($p \leq 0.01$. $p \leq 0.001$) after the treatment period in all the treated groups with MT compared with the control one.

However, we noticed a significant increase ($p \leq 0.001$) of Triglyceride (fig 19) in the groups treated with MT (G3 and G4) compared with the control group.

Urea (fig 20) and Uric acid (fig 21) increased significantly too($p \leq 0.01$. $p \leq 0.001$) in the treated groups G3 and G4 compared with the control group.

In contrast, Albumin concentration (fig 22) decreased in the treated groups with MT compared with the control group.

3.1.5.1.Glucose

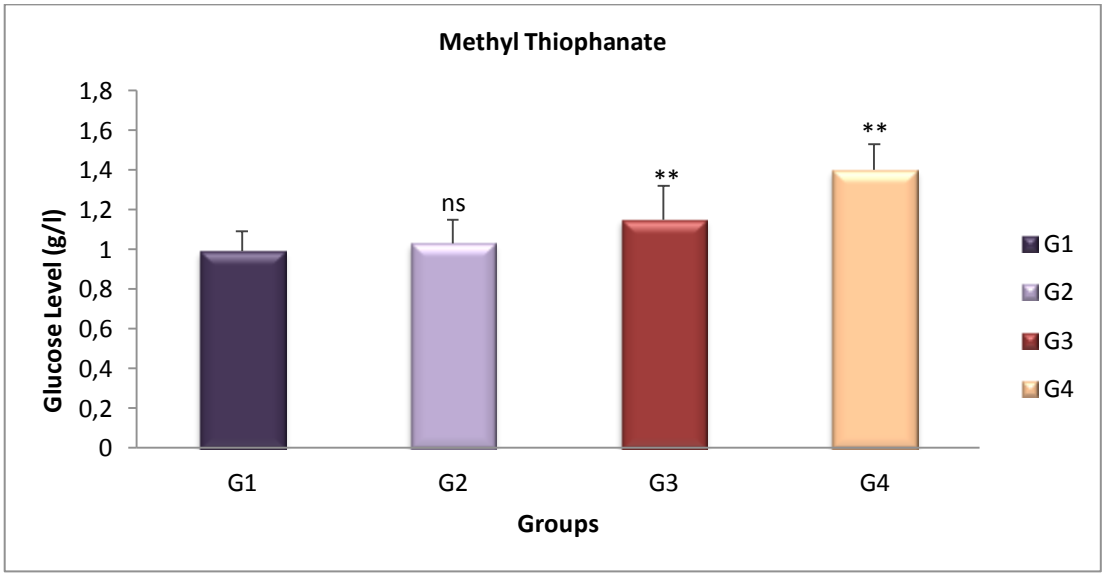

Figure 17: Variation of Glucose level (g/l) of the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**).

3.1.5.2.Cholesterol

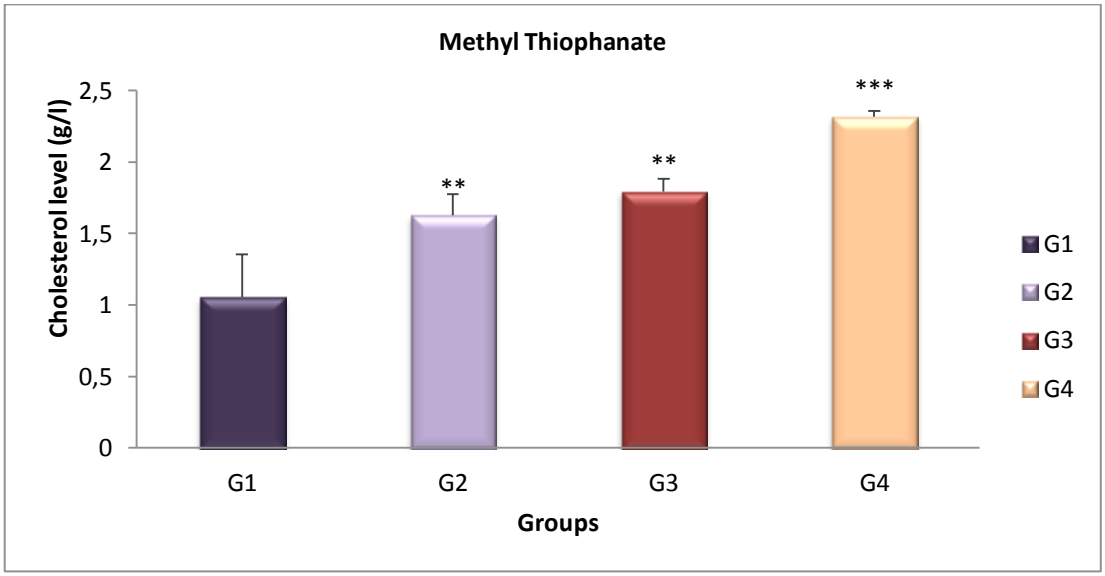

Figure 18. Variation of Cholesterol level(g/l) of the Treated groups and the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**, P≤0.001***)

3.1.5.3.Triglycerides

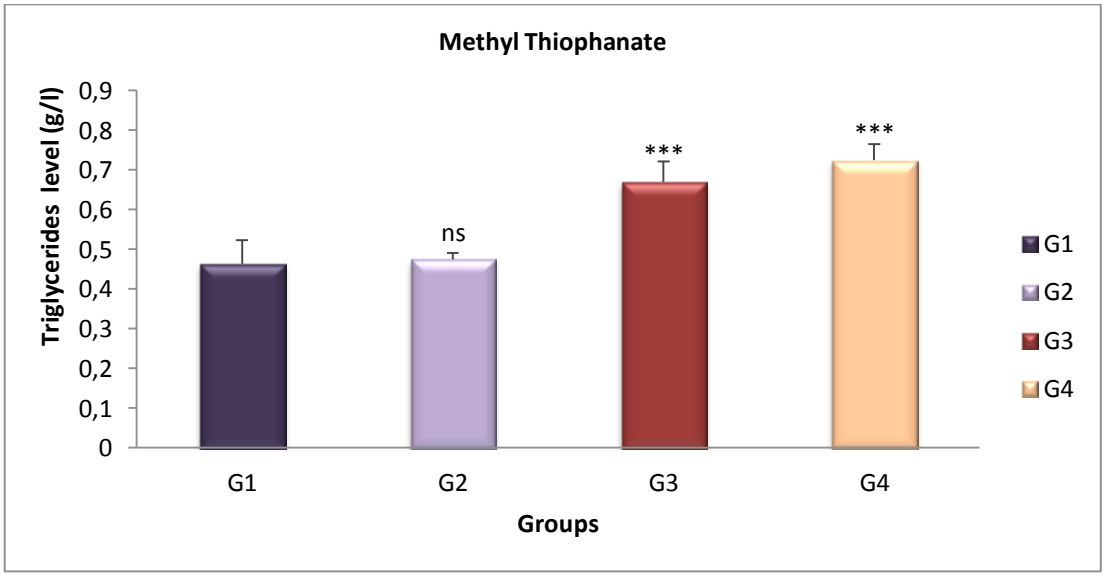

Figure 19. Variation of Triglycerides level (g/l) of the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD, n=6. P≤0.001***).

3.1.5.4. Urea

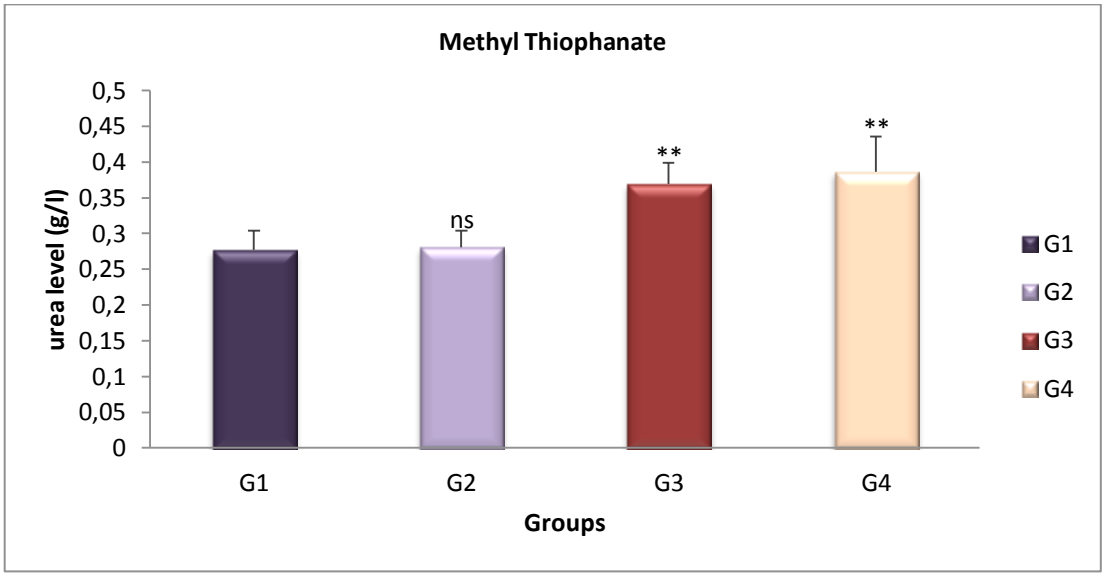

Figure 20. Variation of Urea level (g/l) in the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**)

3.1.5.5. Uric Acid

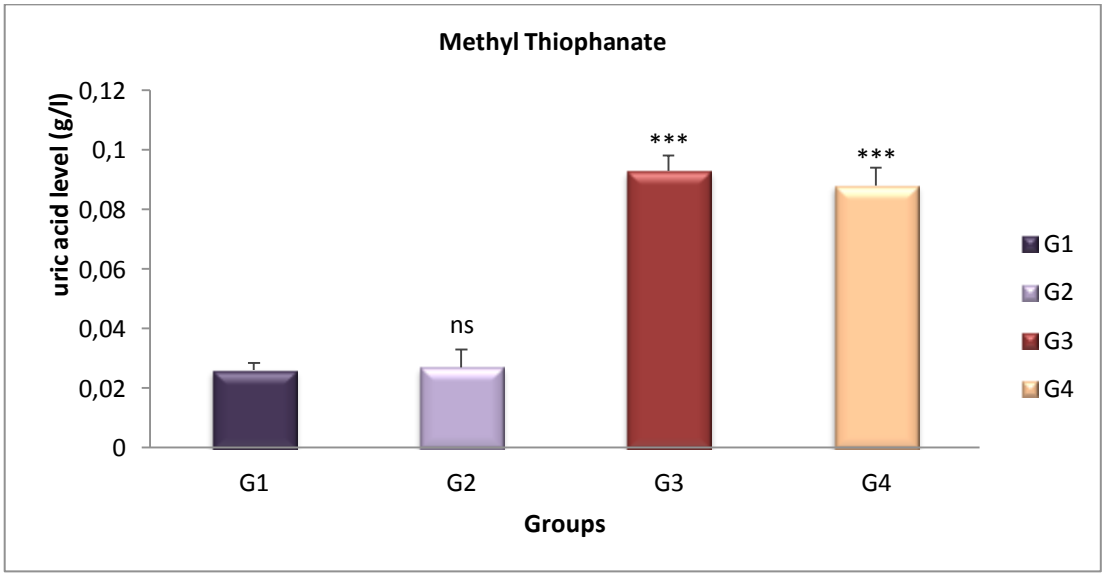

Figure 21. Variation of Uric acid level (g/l) in the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6. P≤0.001***).

3.1.5.6. Albumin

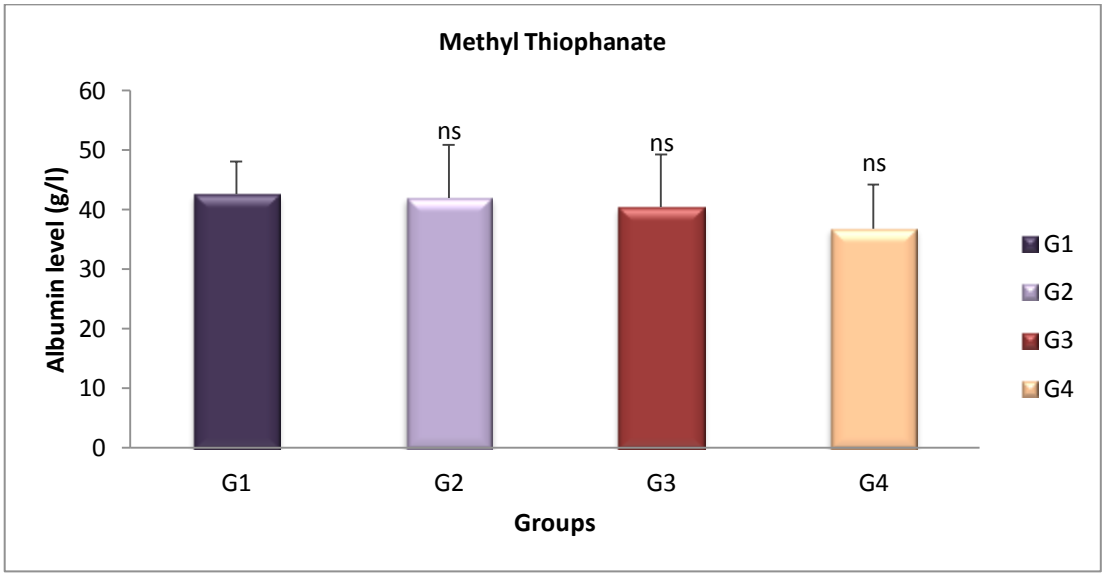

Figure 22. Variation of Albumin level (g/l) in the treated groups with MT compared with the control group after 4 weeks ($\bar{x} \pm$ SD,n=6).

3.1.6. Impact of MT on the Histology of Organs

3.1.6.1. Histology of Testes

Figure 23 shows the histological examination of the different experimental groups. The microscopic examination of testis in the control rabbits (section a) showed typical features of normal seminiferous tubule and their lumen is almost occupied by the mass of spermatozoa.

The sections of testis in treated groups have shown a decrease in the volume of spermatozoa in seminiferous tubules lumens with disorganized arrangements of germ cells from testis section of the treated group with 50mg/kg of MT (section b).

From section (c), it showed dilatation in the seminiferous tubules with a reduced population of germinal epithelium (atrophy) for the group treated with 100 mg/kg of MT.

The maximum of histopatological damage was observed in animals testis section treated with 150 mg/kg of MT (section d). The section revealed clearly a degeneration of cell germ in multiple seminiferous tubules, thus a necrosis and vacuoles are present in affected seminiferous tubules and their lumen are almost empty.

3.1.6.2.Histology of Epididymis

The epididymis microphotographs section (fig 24) of the control group (section a) has shown a normal architecture of the epididymis tubules and their lumen is full of spermatozoa.

For the sections of treated groups, they have shown a considerable decrease of spermatozoa's volume observed in epididymis tubules of the group treated with 50 mg/kg of MT (section b).

Whereas the diameter of empty ducts decreased and become atrophic and necrotic especially for the group treated with the high doses (sections c and d).

3.1.6.3. Histology of Liver

In the control rabbits, liver did not show any pathological changes, they exhibited normal architecture and systematic arrangement of hepatocytes (fig 25, a). In contrast, the rabbits treated with MT showed obvious histopathological changes. The treatment with MT by 50mg/kg induced many histopathological alteration shown in section (b) of liver where hapatocytes were swelled and enlarged and the most of intrahepatic blood vessels were dilated and congested (angioctasis).

section (c) showed that the hepatocytes lost their normal architecture and appearance of cytoplasmic vacuolization and fat vacuols . From the liver section of the high dose of MT, it is shown that the most of the hepatocytes were suffering from necrosis (section d).

3.1.6.4. Histology of Kidney

Kidney microphotographs section (fig 26) of control rabbit (section a) show normal histological appearance**.**

Section (b) of kidney after MT exposure up to 4 weeks at doses of 50 mg/kg revealed tubular dilatation with thickened basement membrane, degeneration changes in cortex region. Along with tubular damage, vacuolated cells are also present besides necrosis in modulary region.

However, these degeneration changes were more severe in kidney section (c) of group (03**)** while it shows hypertrophied and vacuolated cells. The atrophy was observed in cortex region of treated group compared with the control one.

For the group treated with the high dose of MT, the section (d) shows that some Glomerulus have lost their attachment and others were atrophied.

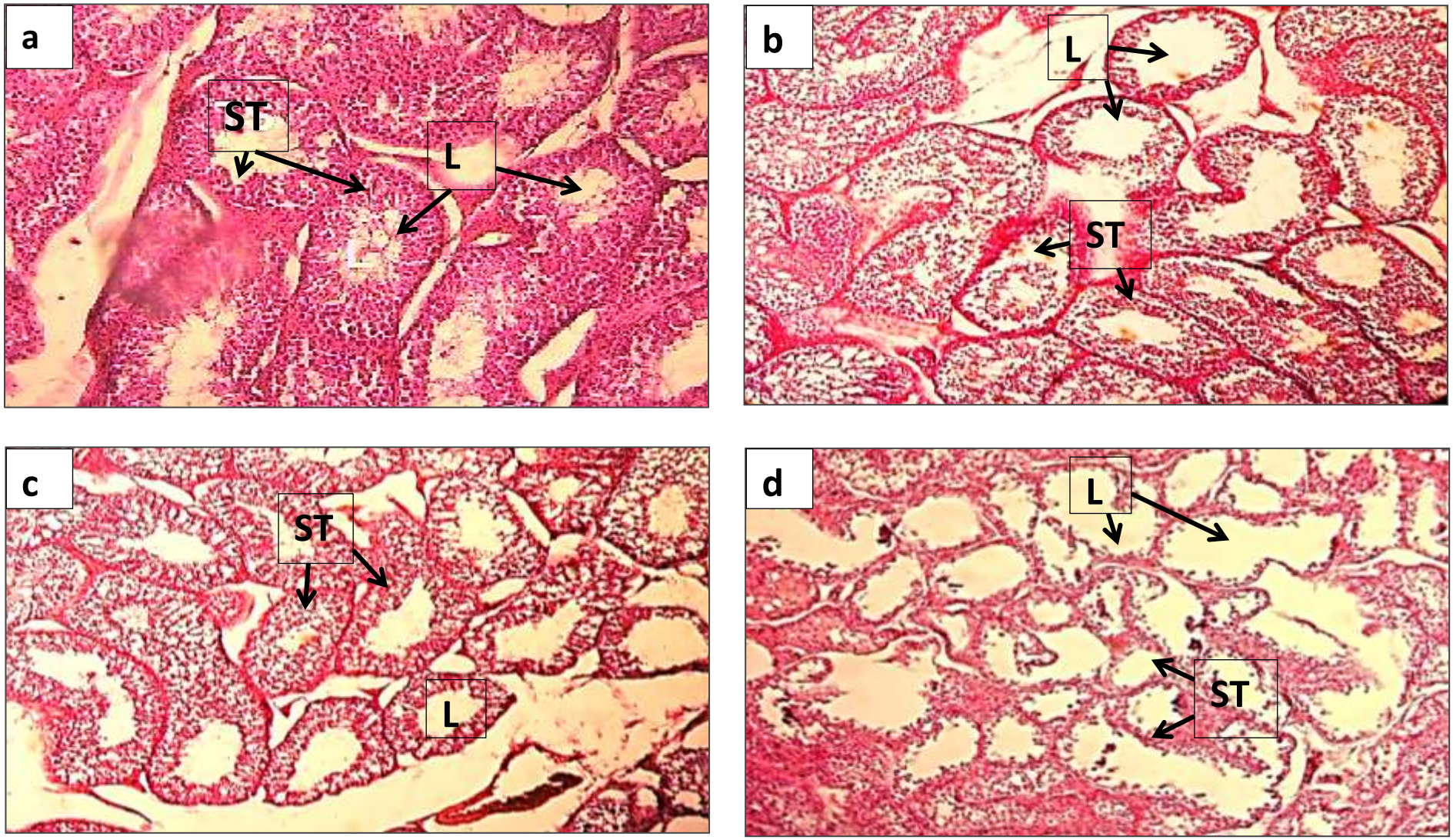

Figure 23. Microphotographs Sections of testes of the experimental rabbits (H&E.300x)/ **a:** control group, **b:** group treated with 50 mg/Kg , **c:** group treated with 100 mg/Kg, **d:** group treated with 150 mg/Kg of MT /**ST:** Seminiferous tubules / **L:** lumen of tubules.

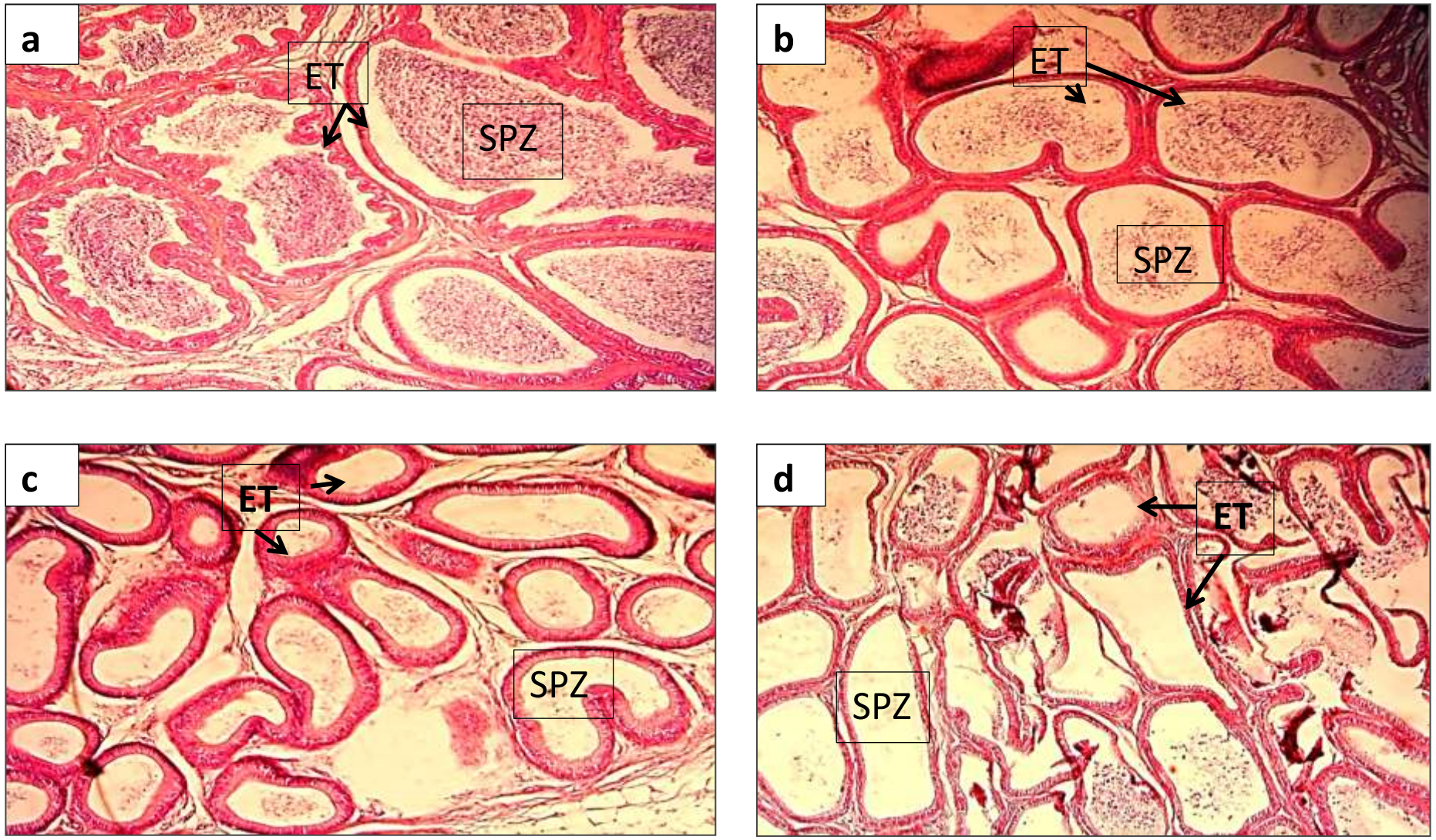

Figure 24. Microphotographs sections of epididymis of experimental rabbits (H&E,300x)/ **a:** control group, **b**: group treated with 50 mg/Kg, **c**: group treated with 100 mg/Kg, **d**: group treated with 150 mg/Kg of MT/ **ET:** Epididymis tubules / **SPZ:** Spermatozoa.

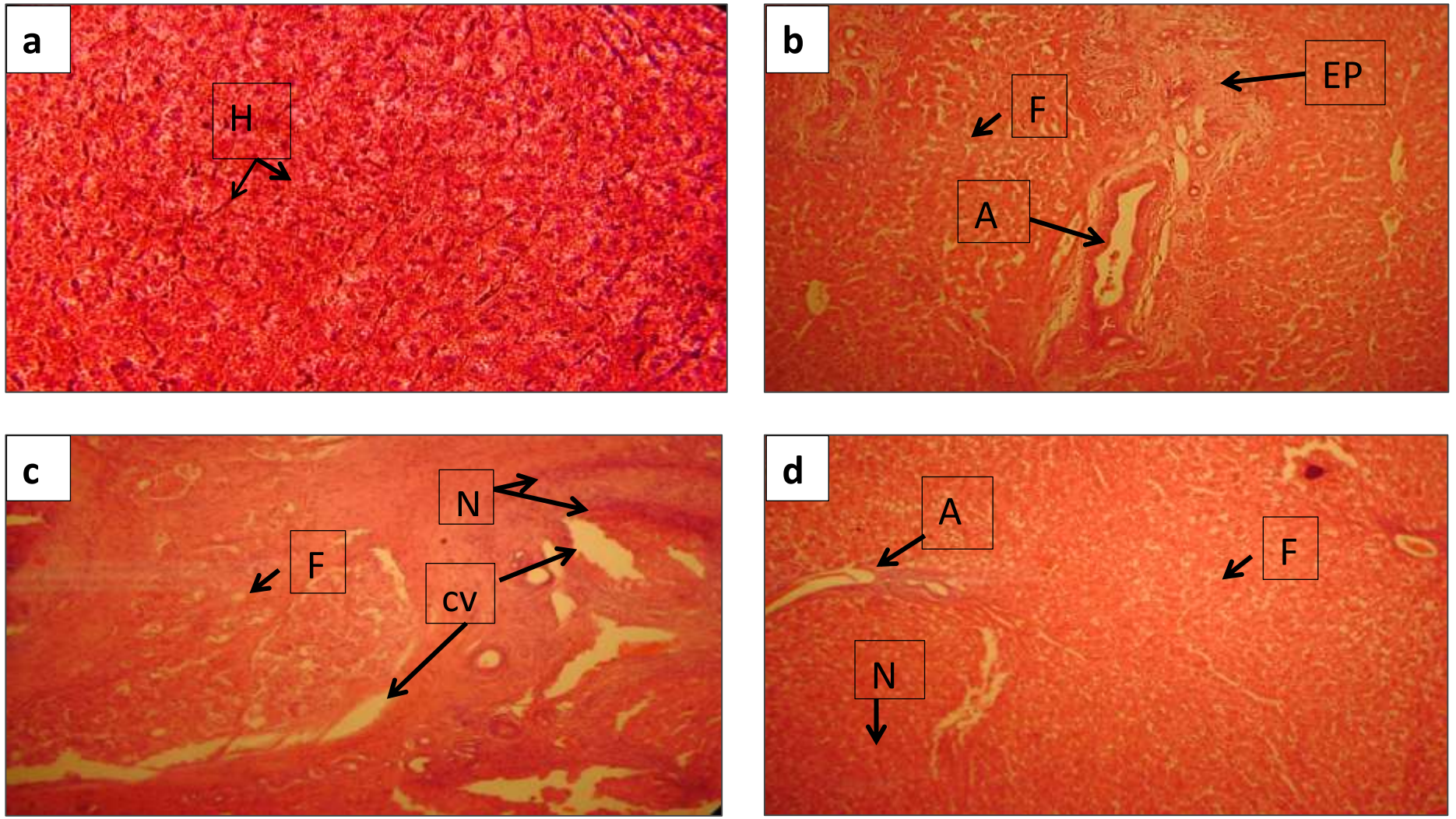

Figure 25. Microphotographs sections of liver of the experimental rabbits (H&E, 150x) / **a:** control group, **b**: group treated with 50 mg/Kg, **c:** group treated with 100 mg/Kg, **d:** group treated with 150 mg/Kg of MT/ **H**: normal hypatocyte cells/**A:** Angiectasis (dilated of blood vessels) / **EP:** endothelial cell proliferation as is seen in Hemangiomas/ **N:** Necrosis / **CV:** cytoplasmic vacuolization/ **F:** fat vacuole.

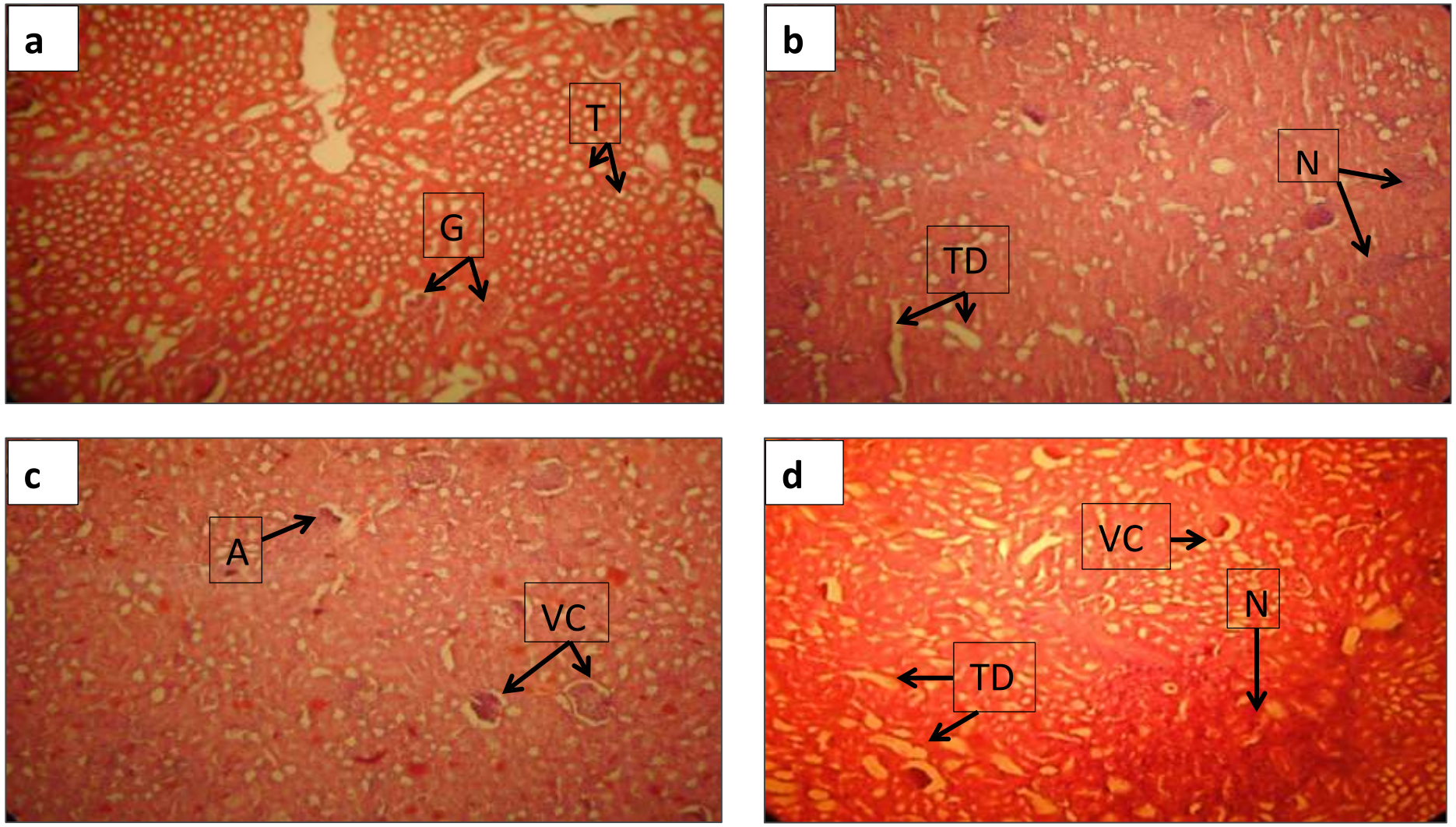

Figure 26. Microphotographs sections of kidney of the experimental rabbits (H&E, 150x)/ **a:** control group, **b:** group treated with 50 mg/Kg , **c:** group treated with 100 mg/Kg , **d:** group treated with 150 mg/Kg of MT/ **G:** Glomerulus/ **T:** Tubules/ **TD:** tubular dilatation/ **N**: necrosis/ **VC:** Vacuolated cells/ **A**: atrophy

3.2. Impact of Thiram During the Second Experimental Protocol:

3.2.1. Experimental Observation of the Effect of Thiram

3.2.1.1. Mortality Level

The table below present percentage of mortality observed for 15 days in the rabbits treated with different doses of Thiram. high level of mortality in rabbits treated with the high dose of Thiram (80 mg/kg) was recorded till it reached 100 % by the end of the week.

Table 06: Percentage of mortality level observed in the treated rabbits with Thiram.

Thiram doses (mg/Kg)	Mortality number	Day	Mortality (%)
G1 (20 mg/kg)	0	/	00%
G2 (30 mg/kg)	0	/	00%
G3 (80 mg/kg)	6	10^{th} , 14^{th},15^{th}	100%

3.2.1.2. Macroscopic Observation of Lung in the experimental groups

Figures (27,a) shows a normal appearance of the lung of the control rabbits. While the macroscopic examinations of lungs of treated animals with 20 and 30 mg/kg of Thiram revealed subpleural and intraparenchymal hemorrhagic spots (fig 27,b,c), On the other hand we examined also one dead rabbit from the group treated with the high dose of thiram and the macroscopic observation shows that the lung suffer from sever hemorrhage presented in fig (27,d).

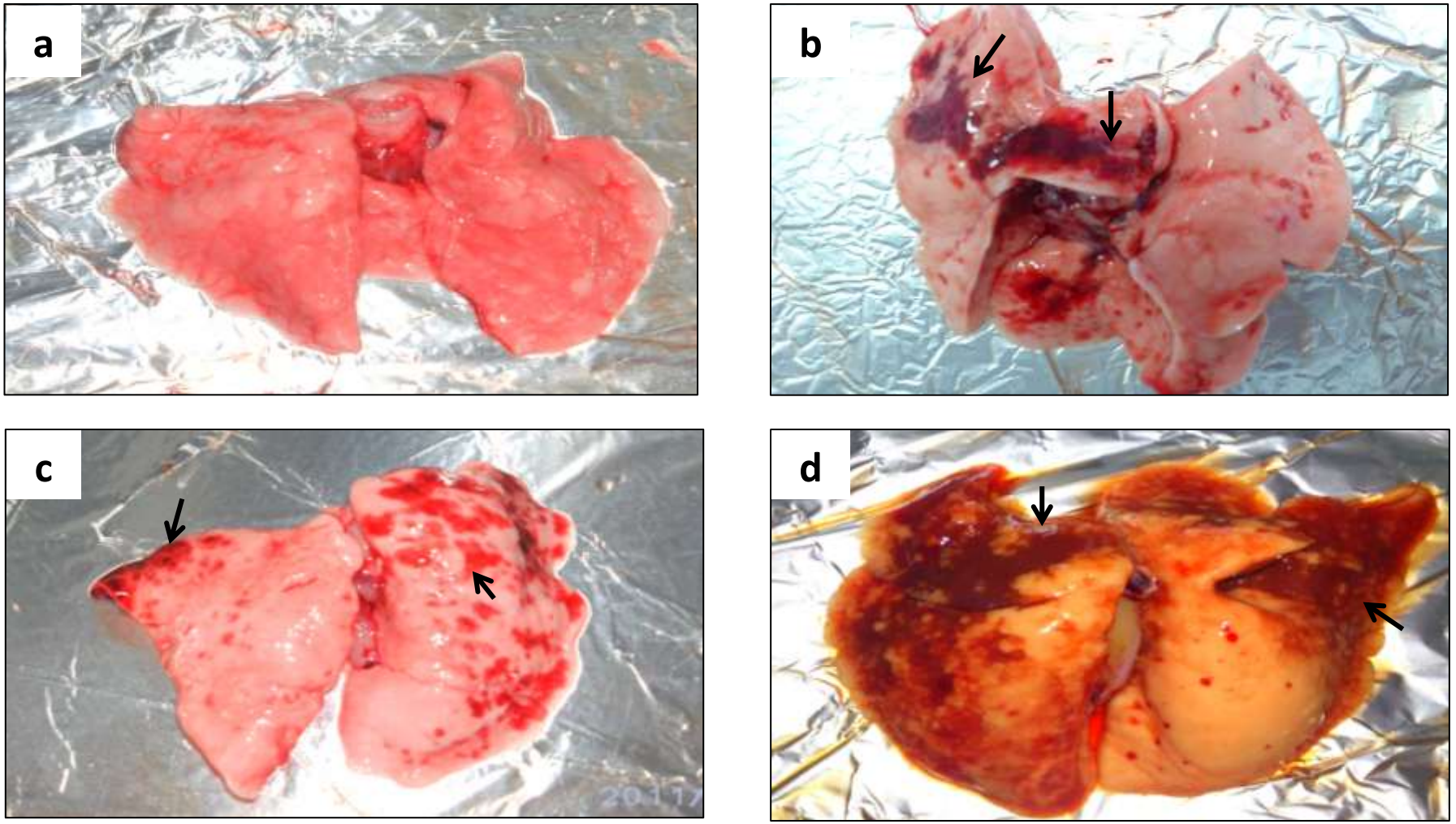

Figure 27. Photographs illustrating the gross morphological of lung of the treated rabbits with Thiram and the control one/ **a:** normal lung of control rabbit, **b:** Treated rabbit with 20 mg/kg of Thiram, **c:** Treated rabbit with 30 mg/kg of Thiram, **d:** lung of dead rabbit from the treated group with 80 mg/kg of Thiram. **Arrows:** Subpleural and intraparenchymal hemorrhagic spots.

3.2.1.1.Variation of Organs Weight

The result revealed in table (07) that the treatment with Thiram for 15 days not affects significantly the liver and kidney weight. However the testes and epididymis weight were significantly decreased ($p \leq 0.01$. $p \leq 0.001$) in the groups treated with Thiram as compared with the control.

Table 07: Weight of the liver, kidney, testes and epididymis of the treated rabbits with Thiram compared with the control group after 2 weeks (n=6).

Weight (g)	G1: Control	G2: 20mg/kg	G3: 30mg/kg
Liver	54.85±3.54	55.11±2.25	56.23±1.4
Kidney	5.65±0.70	5.19±0.82	5.42±0.63
Testes	2.27±0.2	1.09±0.1***	1.27±0.06**
Epididymis	0.50±0.11	0.22±0.05***	0.26±0.02***

($\bar{x} \pm$ SD, P≤0.05*, P≤0. 01**, P≤0.001***) compared to controls.

3.2.2. Impact of Thiram on Fertility

3.2.2.1. Spermatozoas Count :

The study of epididymal sperm shows that sperm count (Fig 28) have significantly decreased (P≤0.001) in the treated animals compared with the control group.

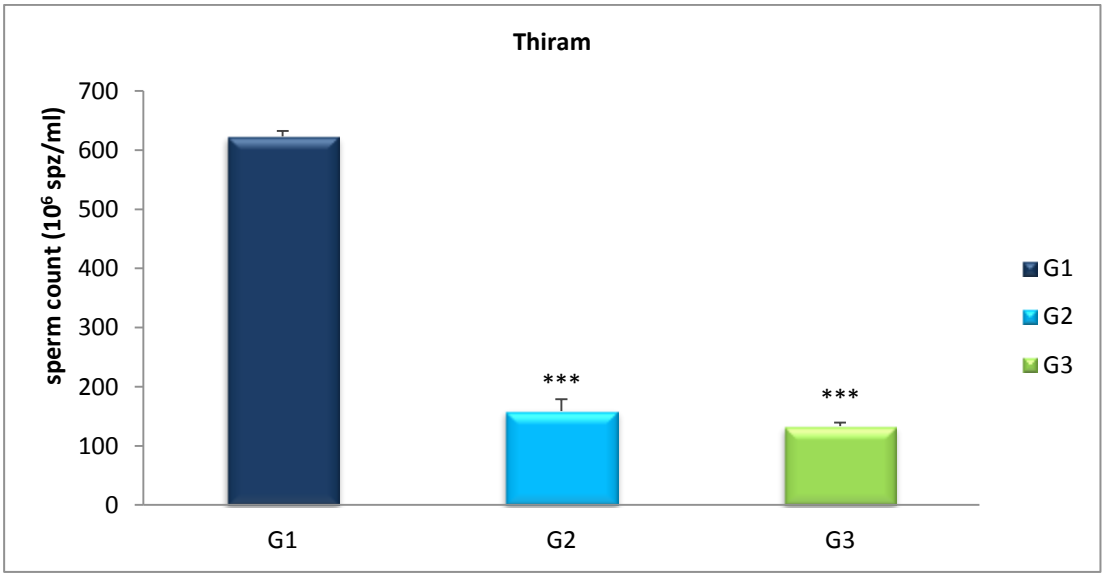

Figure 28. Epididymal sperm count (x10^6/ml) of treated groups compared with the control one after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.001***).

3.2.2.2. Motility of Spermatozoa:

The result shows that the sperm motility in (Fig 29) have significantly decreased (P≤0.001) in the treated animals as compared to the control.

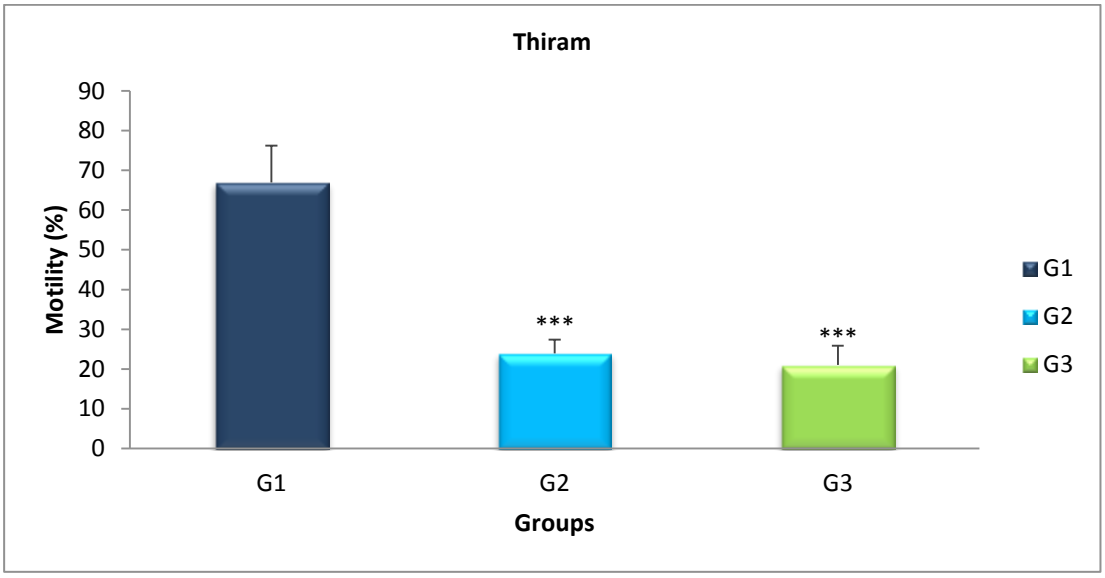

Figure 29. Epididymis sperm motility (%) of treated groups with Thiram compared with the control one after 2 weeks ($\bar{x}$ ± SD,n=6. P≤0.001***)

3.2.2.3. Speed of Spermatozoa:

Speed of spermatozoa also affected in the treated animals (fig 30) when the result shows a high significant decrease of spermatozoa speed in the treated groups compared with the control.

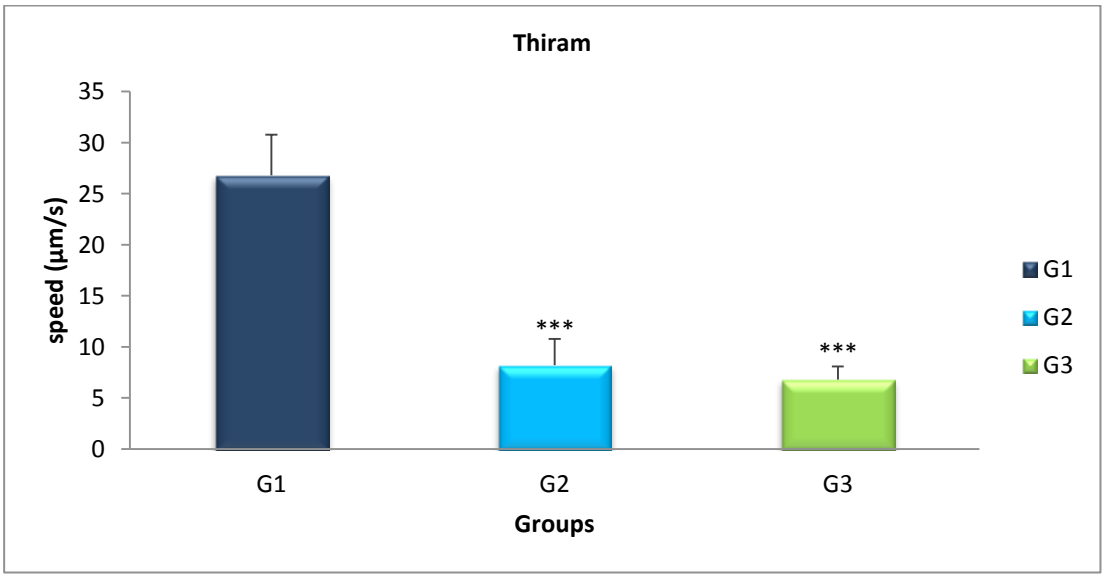

Figure 30. Epididymis sperm speed (µm/s) of treated groups with Thiram compared with the control one after 2 weeks ($\bar{x}$ ± SD,n=6. P≤0.001***).

3.2.2.4. Viability of Spermatozoa

- **Eosin Trial:**

The Fig 31 also indicates a decrease in the percentage of alive spermatozoa with an increase of dead spermatozoa in the treated groups with Thiram compared with the control group.

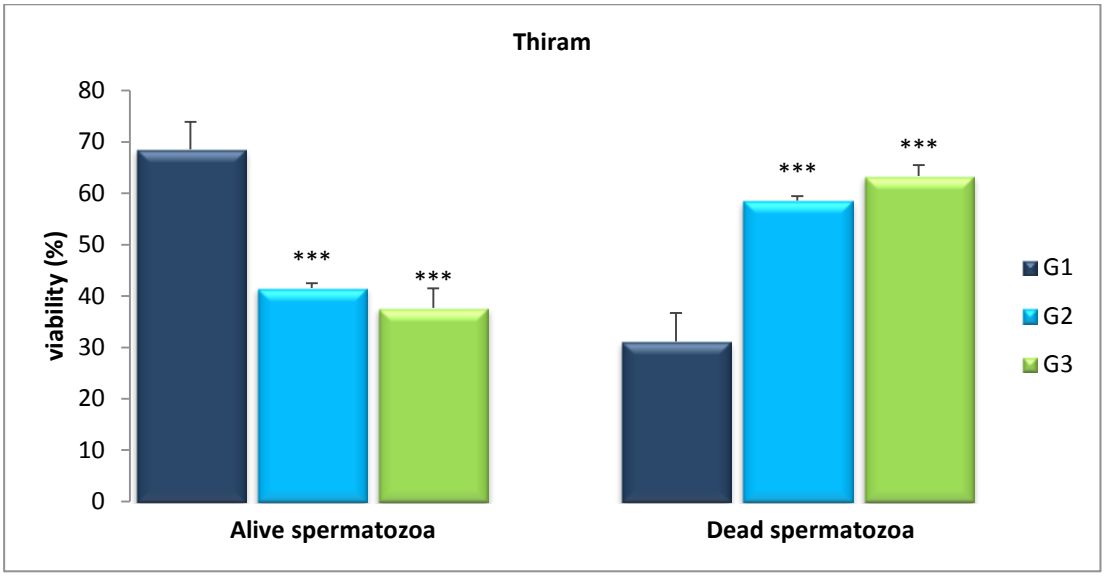

Figure 31. Epididymal sperm viability (%) of the treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.001***).

- **Hypo-osmotic Swelling Test**

Figure (32) shows a significant increase (p≤0.01) of dead spermatozoa (N) in the treated group compared with the control one.

The percentage of alive spermatozoa type (A) significantly decreased (p≤0.05, p≤0.01) in the experimental groups compared to the control one.

The percentage of alive spermatozoa type (B) significantly reduced (p≤0.05, p≤0.01) in the treated groups compared with the control one.

Also, we noted a significant decrease in percentage of alive spermatozoa type (C) (p≤0.05) in the treated groups compared with the control one.

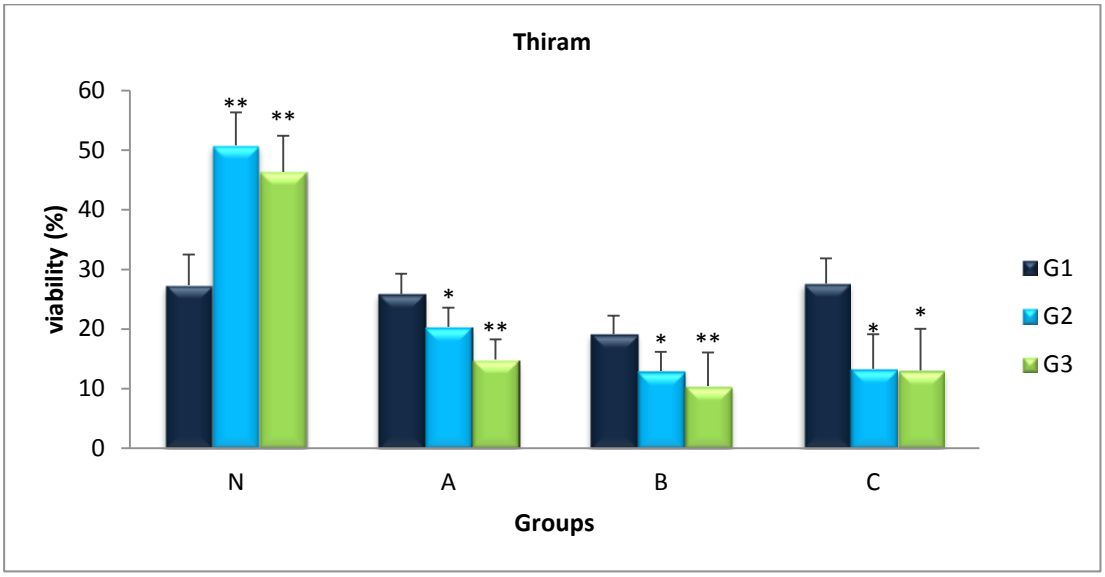

Figure 32. Epididymal sperm viability (%) of treated groups with Thiram compared with the control after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*, P≤0.01**)

3.2.2.5. Plasma Testosterone Level

The results revealed in Figure (33) that the testosterone level significantly reduced (P≤0.001) in Thiram treated rabbits when compared with the control animals.

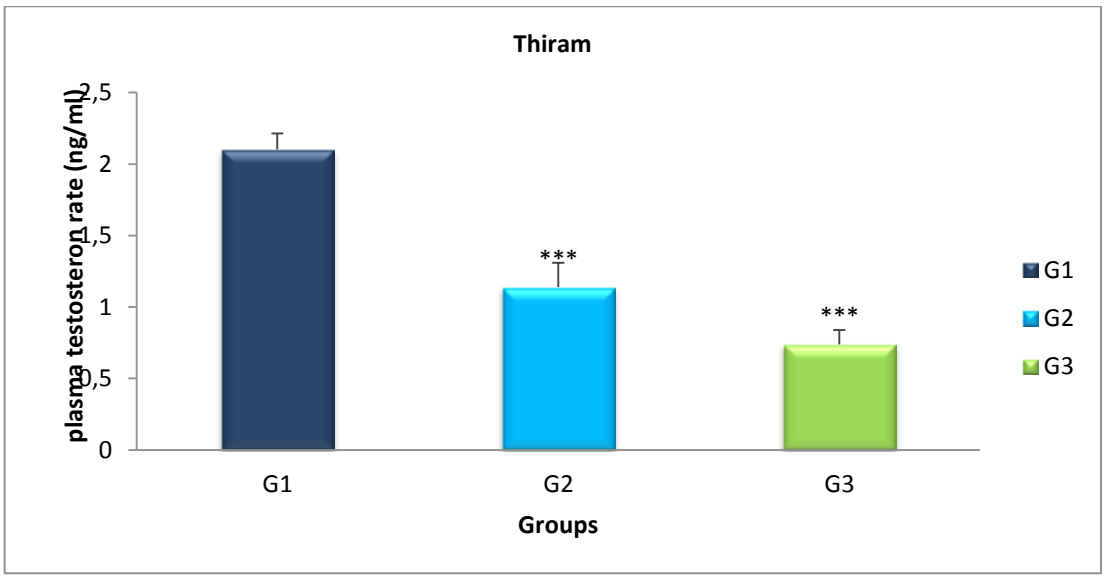

Figure 33. Variation of testosterone level in treated groups by Thiram compared with the control group after 2 weeks (X±SD,n=6. P≤0.001***).

3.2.3. Impact of Thiram on Hematological Parameters

Data shows that red blood cells and hematocrit significantly decreased ($p \leq 0.05$) in the group treated with 30 mg/kg of Thiram compared with the control group, while hemoglobin increased insignificantly in all the treated groups.

White blood count significantly increased ($p \leq 0.05$) in the group treated with 30 mg/kg compared with the control group.

Whereas Platelets significantly decreased ($p \leq 0.01$) in the treated groups compared with the control group. On the other hand MCV, MCH were not significantly affected at all the treated groups. MCHC significanty decreased in the group treated with 30 mg/kg compared with the control one.

Table 08: Variation of Hematological parameters of treated groups with Thiram compared with the control one after 2 weeks (n=6).

Parameters	**G1/ control**	**G2/ 20mg/kg**	**G3/ 30mg/kg**
RBC (10^{12}/l)	6.15±0.81	5.66±1.25	4.39±0.57*****
WBC (10^{9}/l)	6.89±1.22	8.91±1.68	10.16±0.75*****
HGB (g/dl)	115.67±8.74	124.33±8.39	127.67±3.21
HCT (%)	35.3±3.1	32.07±2	31.17±1.26*****
PLT	261±19.3	141.7±20******	128±32.9******
MCV	58,75±3,59	56±3.61	55.67±1.53
MCH	15.75±3.7	18.07±1.19	15.87±3.2
MCHC	328±30.9	322.7±10.1	287±63.2*

($\bar{x} \pm$ SD, P≤0.05*, P≤0.01**)

3.2.4. Impact of Thiram on Thyroids Hormones

Figures 34 and 35 show clearly a significant decrease ($p \leq 0.05$, $p \leq 0.01$) of thyroids hormones level T3 (fig 34) and T4 (fig 35) in the treated rabbits compared with the control rabbits.

3.2.4.1. Tri-iodothyronine (T3)

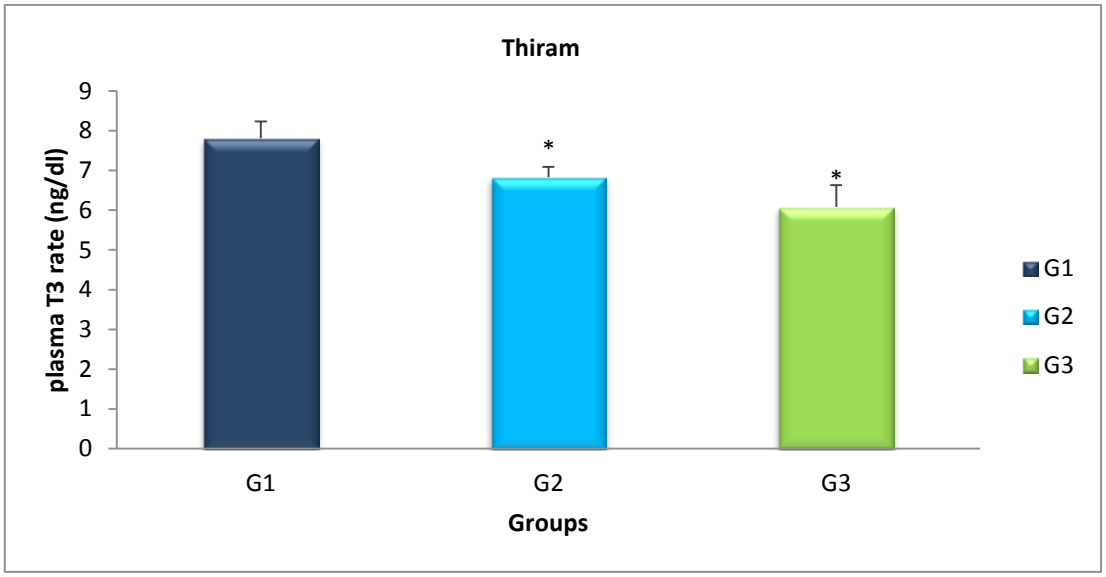

Figure 34. Variations of tri-iodothyronine level (ng/ml) in the Treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*).

3.2.4.2. Thyroxin (T4)

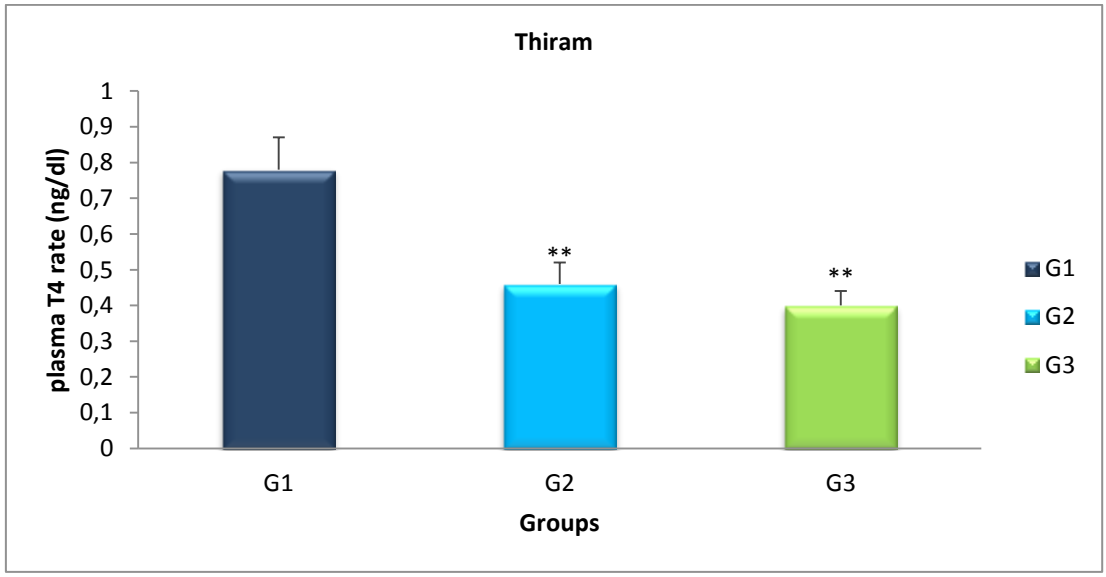

Figure 35. Variations of Thyroxin level (ng/ml) in the treated groups with Thiram and the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**)

3.2.5. Impact of Thiram on Biochemical Parameters

The obtained results have shown that the Glucose (fig 36) , Cholesterol (fig 37) and Triglyceride (fig 38) levels significantly increased ($p \leq 0.01$) in the treated groups with Thiram compared with the control.

Urea (fig 39) and Uric acid (fig 40) levels also significantly increased ($p \leq 0.01$. $p \leq 0.05$) in the treated group with 30mg/kg of Thiram compared with the control.

Whereas Albumin level (fig 41) decreased in the treated groups with Thiram compared with the control.

3.2.5.1. Glucose

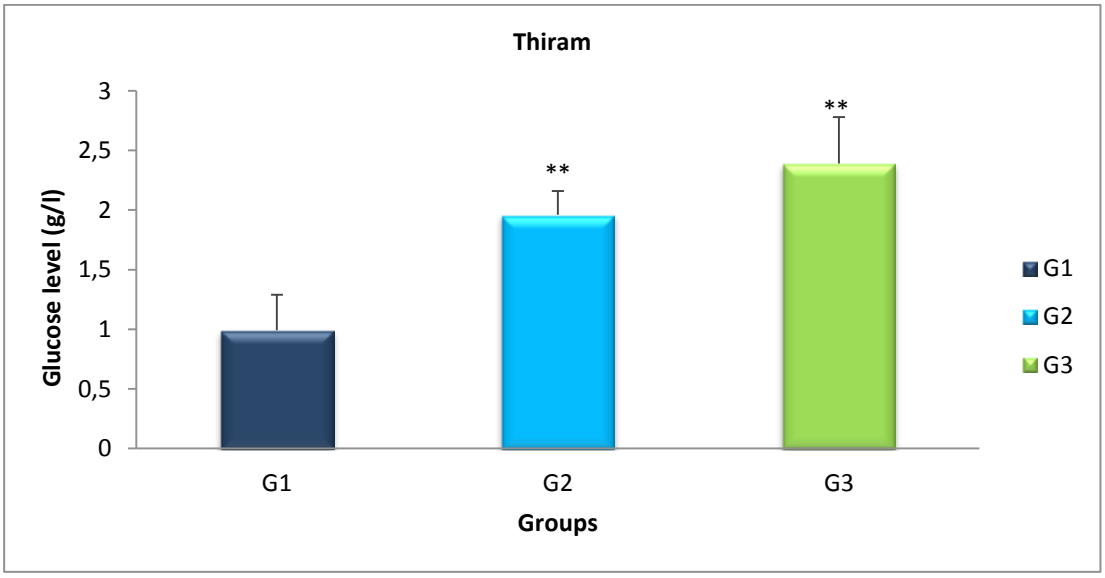

Figure 36.Variation of Glucose level (g/l) in the treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm SD$,n=6. P≤0.01**).

3.2.5.2. Cholesterol

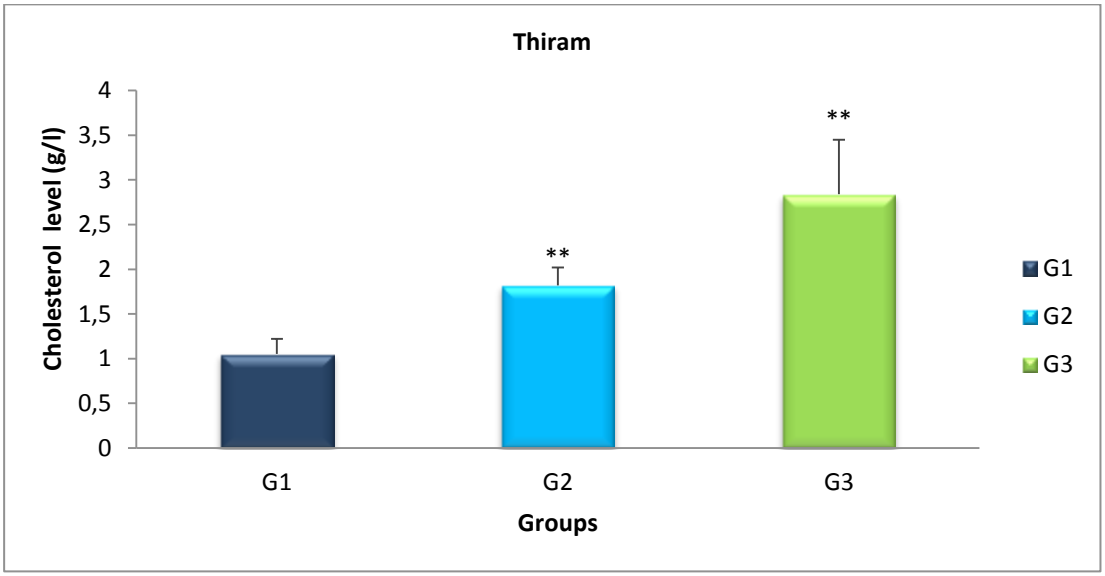

Figure 37. Variation of Cholesterol level (g/l) in the treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**).

3.2.5.3. Triglycerides

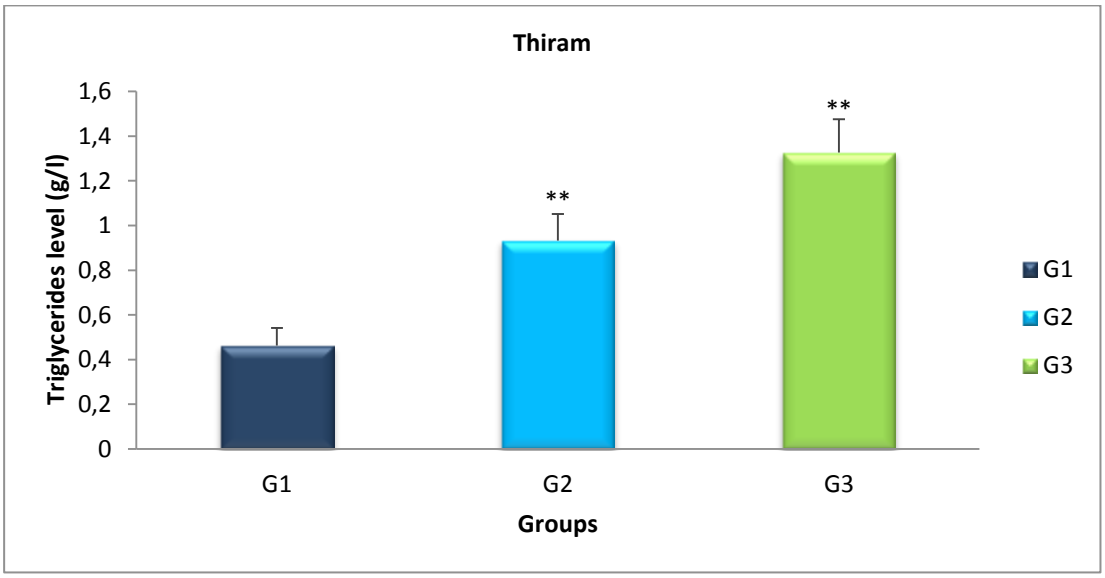

Figure 38. Variation of Triglycerides level (g/l) in the Treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm SD$,n=6. P≤0.01**).

3.2.5.4. Urea:

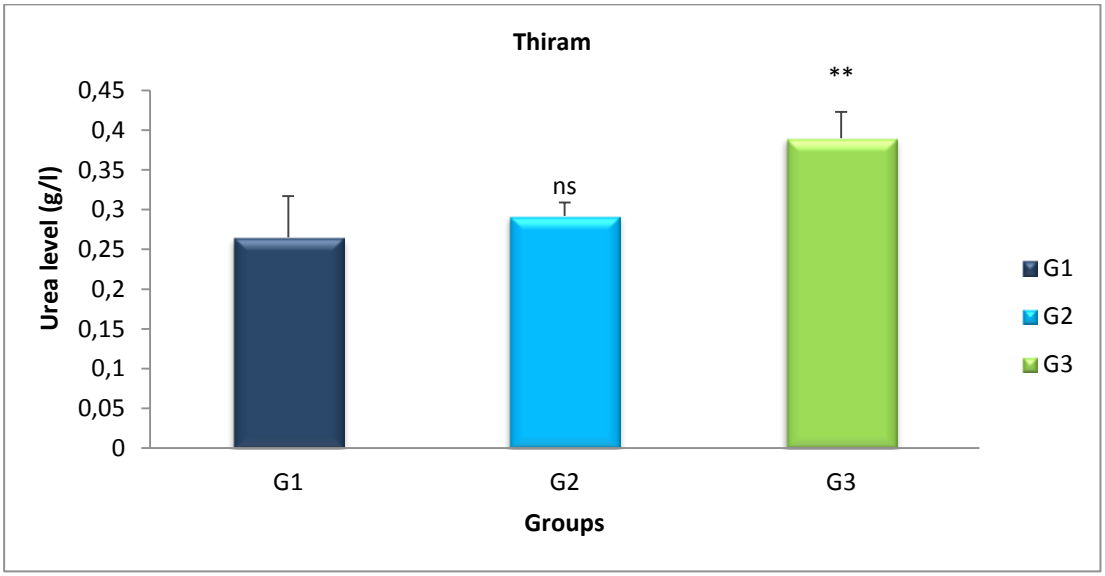

Figure 39. Variation of Urea level (g/l) in the Treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.01**).

3.2.5.5. Uric acid

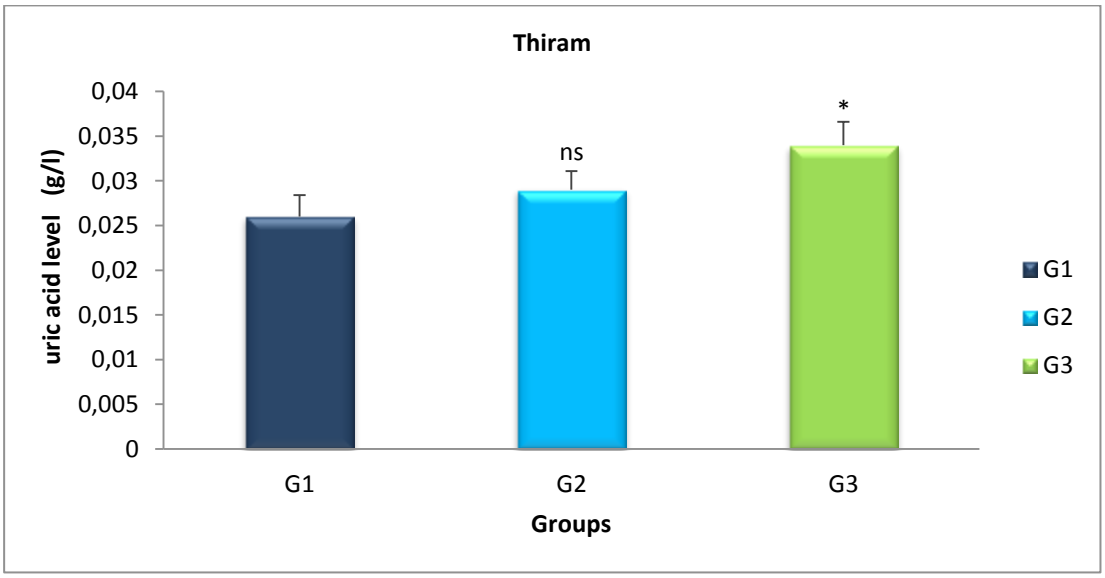

Figure 40. Variation of Uric acid level (g/l) in the Treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*).

3.2.5.6. Albumin

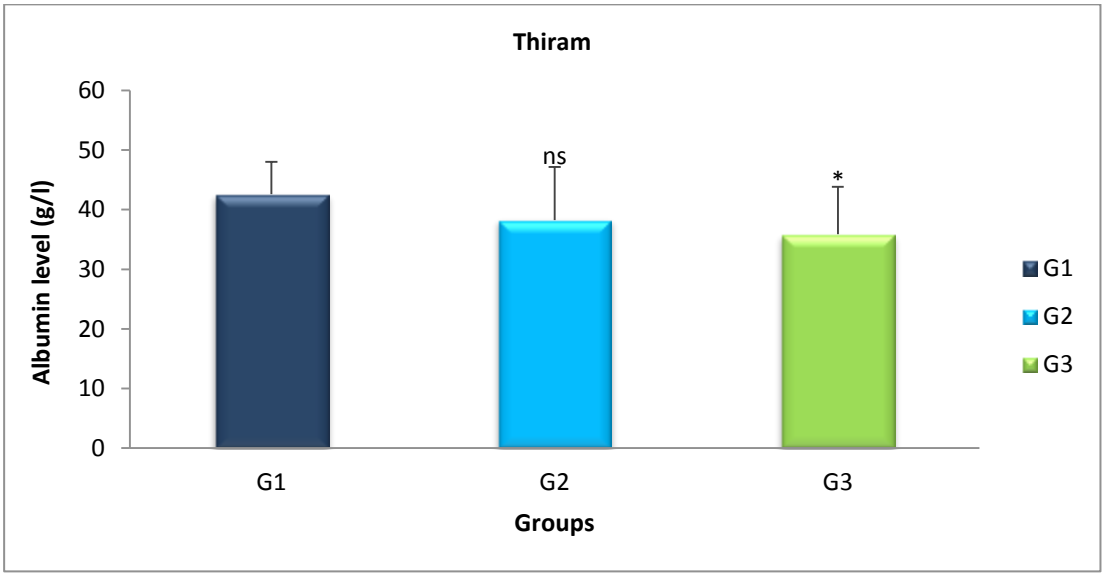

Figure 41. Variation of Albumin level (g/l) in the Treated groups with Thiram compared with the control group after 2 weeks ($\bar{x} \pm$ SD,n=6. P≤0.05*).

3.2.7. Impact of Thiram on the Histology of Organs

3.2.7.1. Histology of testes

Histopathological changes of the testes assessed by light microscopy of tissue sections with H&E staining. The testes micrographs sections (fig 42) of the control rabbits showed a normal structure of seminiferous tubules with all the successive stages of spermatogenesis (a). In contrast a marked histopathological changes were observed in the seminiferous tubules after Thiram treatment in rabbit. These changes include damaged seminiferous tubules, which showed the lumen contained cellular debris and is almost devoid of sperms (b,c).

3.2.7.2. Histology of epididymis

The micrographs sections (fig 43) of control group (a) shows a normal morphology of epididymis tubules where has full with the mass of mature spermatozoa and has no physiological abnormalities. On the other hand, sections of the treated groups shows that the epididymal tubules are small compared with the control section, also it was almost empty of the mass of spermatozoa with abnormal architecture of epididymal tissue (b,c).

3.2.7.3. Histology of liver

Fig (44) present the histopathologic changes of liver, microphotographs section (a) show that the of liver of control rabbit exhibited normal architecture and systematic arrangement of hepatocytes.

As shown in (Fig 44, b), Thiram exposure with dose 20 mg/kg caused liver injury as indicated by necrosis and presence of focal nodular hyperplasia, while the blood tubules dilated. about (Fig 44, c) almost the same criteria of liver treated by 20 mg/kg has shown in the section of liver treated by 30 mg/kg (necrosis, focal nodular hyperplasia).

3.2.7.4. Histology of kidney

Kidney is the most important organ of filtration and metabolism of xenobiotic so the section of the control group (fig 45, a) shows a normal architechter in the histology of kidney .On the other hand microphotographs section shown in figure (45, b,c) present the histology of the group treated with 20 mg/kg and 30 mg/kg of thiram. The sections indicates that thiram causes abnormal architecture of kidney tissue (tubular damage, vacuolated cells and necrosis).

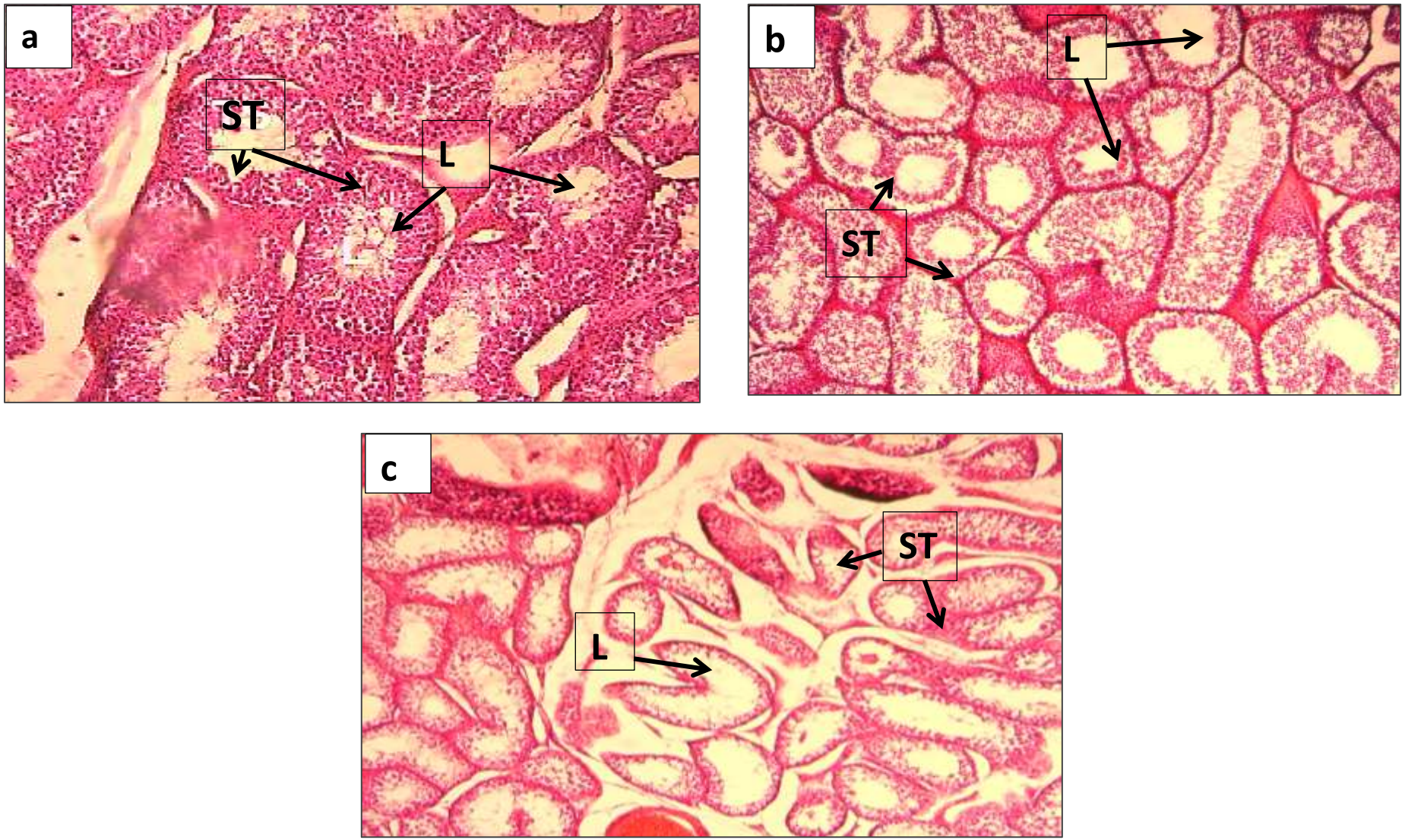

Figure 42. Photomicrographs sections of testes of the experimental rabbits (H&E, 300x)/ **a:** control group, **b:** group treated with Thiram (20 mg/Kg), **c:** group treated with Thiram (30 mg/Kg)/ **ST:** seminiferous tubules. **L:** lumen of tubules. .

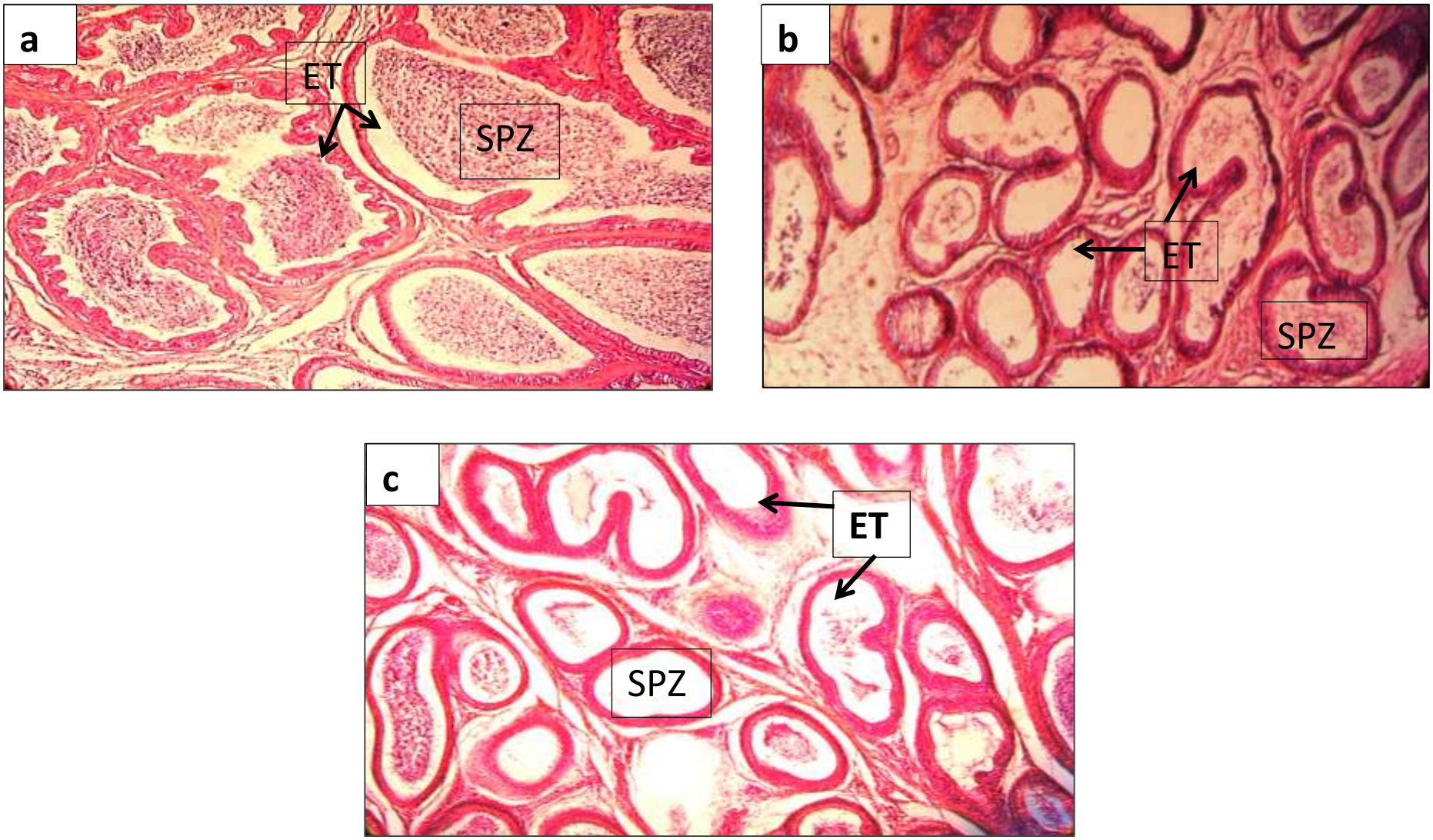

Figure 43. Microphotographs sections of epididymis of the experimental rabbits (H&E, 300x), **a:** control group, **b:** group treated with Thiram (20 mg/Kg), **c:** group treated with Thiram (30 mg/Kg)/ **ET:** epididymal tubules/ **SPZ:** spermatozoa.

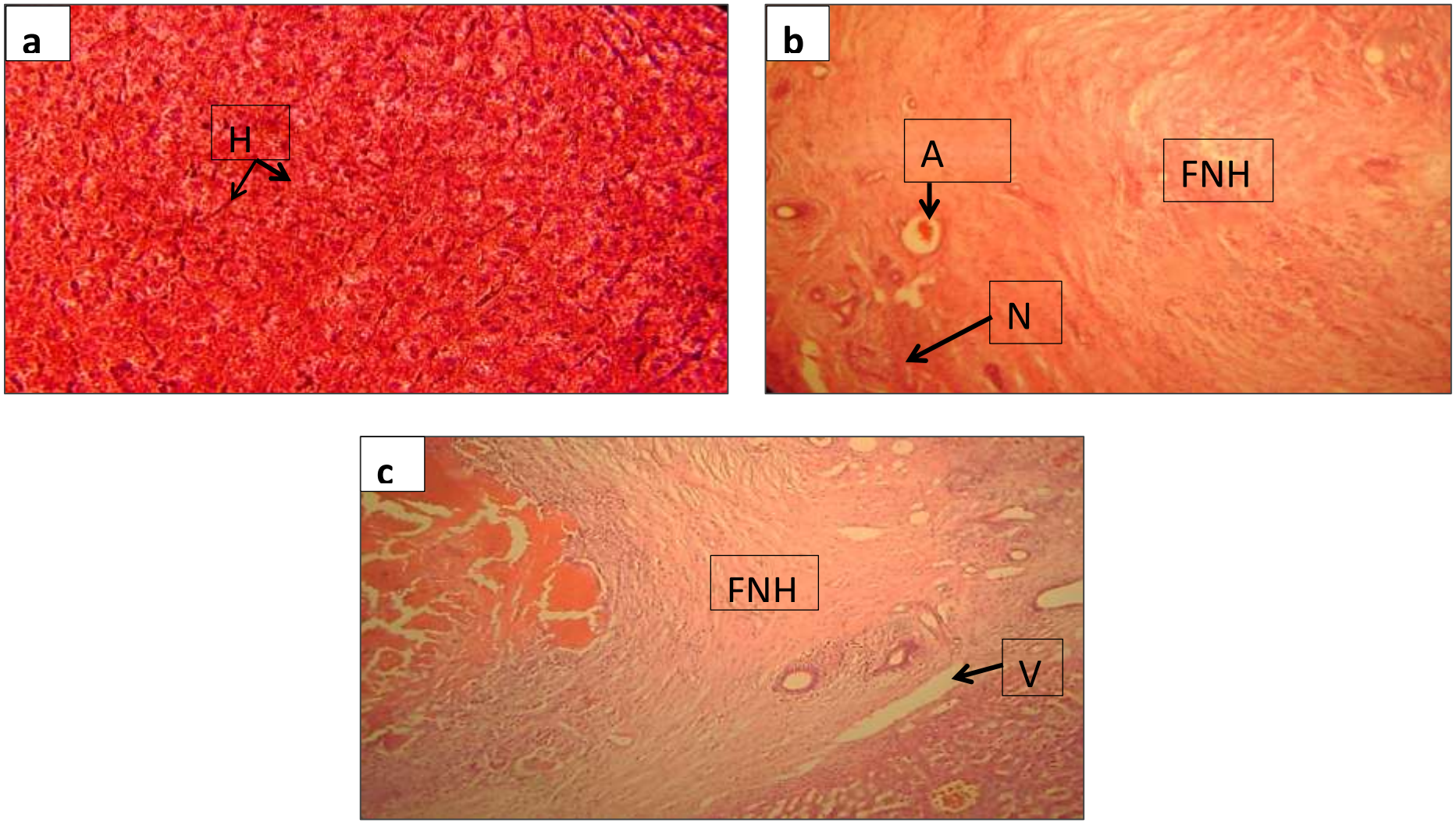

Figure 44. Microphotographs sections of liver of the experimental rabbits (H&E, 150x)/ **a:** control group, **b:** group treated with Thiram (20 mg/Kg), **c:** group treated with Thiram (30 mg/Kg)/ **H:** normal hepatocyte cells. **A:** dilatation of blood tubules. **FNH:** focal nodular hyperplasia. **V**: vacuole

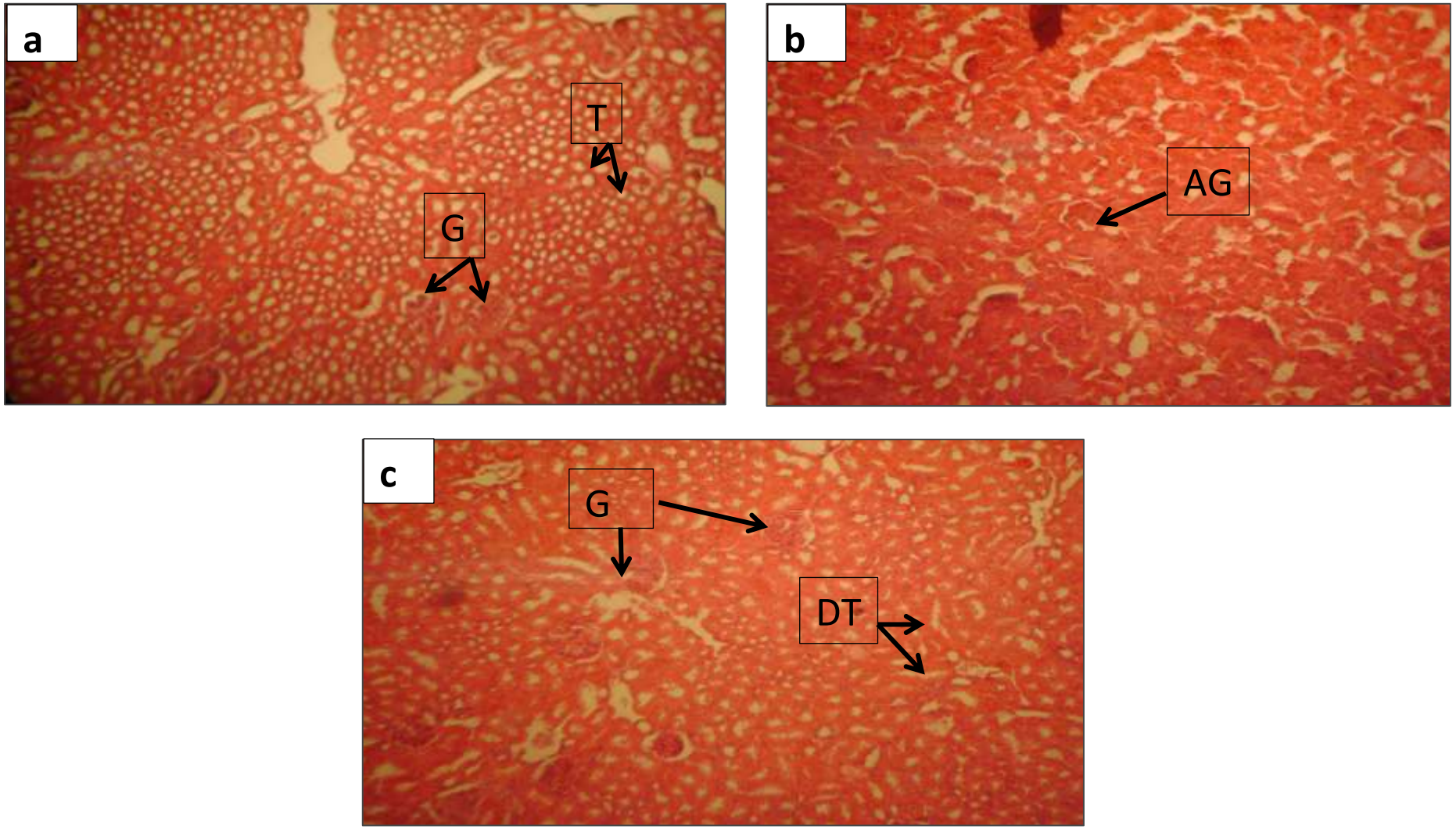

Figure 45. Microphotographs sections of kidney of the experimental rabbits (H&E, 150x)/ **a:** control group, **b:** group treated with Thiram (20 mg/Kg), **c:** group treated with Thiram (30 mg/Kg) /**G:** glomerulus/ **T:** tubules/ **AG:** atrophic glomerulus/ **DT:** dilated tubules.

Discussion

4. DISCUSSION

4.1. Impact of Methyl Thiophanate

Investigations reported that Methyl Thiophante is recognized as reprotoxic agent fungicide. MT can negatively affect the human and animal health especially on reproductive system and can include an altered sexual behavior, altered fertility and problems with sperm shape or count **(Adedara et *al*., 2013).**

The results of our study show a considerable decrease in testis weight and epididymis of the treated rabbits with MT compared to control group. The decrease of the testis weight can be explained by the susceptible effects of MT on histological constitution and morphology of Sertoli cells. On the other hand, it is admitted that the gonads are the target bodies of pesticides **(Kojima et *al*., 1992).** A similar study has shown the same observations on the rats treated with Carbendazim **(Lim et *al*., 1997).**

Exposure to pesticides interferes with spermatogenesis by damaging the testes. Several papers have reported that treatment of experimental animals with the fungicide Carbendazime (MBC) brings about disruption of spermatogenesis and may even temporarily lead to decrease fertility by modifying sperm count, structure, motility and viability of spermatozoa **(Hess et *al*., 2000; Akbarsha et *al*., 2000).**

The present study demonstrates that exposure to MT reduces the sperm count in all the treated groups compared with the control one. The same result was obtained by another study **(Lamfon, 2012).** The observed reduction in the number of sperm seems to be a consequence of a direct alteration on testicular cells, as a similar study on rats indicated that the used fungicide MT caused dysfunction in the spermatogenesis and Sertoli cells **(Markelewicz et *al*., 2004),** or indirect via the hypothalamic-pituitary axis by disturbing the secretion of GnRH which stimulates adeno-hypophysis to secrete LH and FSH by modifying the receiving sites affecting testosterone secretion **(WWF, 1998; Toppari, 2008).**

This hypothesis can also explain the decrease of the speed and motility of the spermatozoa in the treated groups, especially those treated with high doses of MT as compared to the control group. A similar study with carbendazim administered to male *Wistar* rats reported

that the motility of spermatozoa is due to the flagellar beat which, in turn, is dependent to the microtubular apparatus of the flagellum, as MBC is a microtubular poison. MBC through testicular and epididymal routes would affect the sperm flagellar microtubules, which leads to sperm motility impairing **(Akbarsha et *al.*, 2001).**

Sertoli cell is one of the elongated striated cells lining the seminiferous tubules that support and nourish the spermatids. It helps in the process of spermatogenesis thus the production of sperm, **(Yan et *al.*, 2000, Li et *al.*, 2012)**. This is further supported by a decrease in sperm viability in our results where the percentage of the alive sperm was declined in the treated animals compared with the control group. It is well documented that carbendazim alters sertoli cells function, and it was found to cause adverse effects such as sloughing of germ cells **(Gray et *al.*,1994)** and inhibition of germ cell division **(Nakai et *al.*, 2002).**

The present data show that in all tested doses there is a large decrease in plasma testosterone levels after MT treatment. Reduction in testosterone levels has been found by several studies in mammals **(Friedmann et *al.*, 2002)** as well as reptiles **(Crain et *al.*, 1997)**, amphibians **(Hayes et *al.*, 2002)** and fish species **(Spano et *al.*, 2004)**. Supporting earlier data **(Trentacoste et *al.*, 2001),** decrease of testosterone level may be a result of the effect of MT in Leydig cells production and/or metabolism. This point of view is supported by the present data, showing alterations in the histology of testis as well as alteration in seminiferous tubules and consequently an alteration in the morphology of Leydig cells in the treated animals.

Therefore, it is likely that the MT effects on reproductive function may be not only direct on testicular steroidogenesis but also indirect via hypothalamic-pituitary-gonadal axis **(Laws et *al.*, 2009).** The same results obtained in this research is going in the same direction as one obtained by **Costa et *al.* (2010)** who demonstrated that the treatments with Atrazine (50 mg/kg, 200 mg/kg and 300 mg/kg) in adult rats reduce testosterone levels. Thus Atrazine affects the testicular androgenesis and leads to changes in spermatogenesis**.**

In regards to the histology of reproductive organs, we reported that MT causes histopathological changes which observed in epididymis and testes.

The sections of the testis in the treated animals with MT show a significant decrease in diameters of the seminiferous tubules. Hence, histological microphotographs revealed that the spermatogenic cells were degenerated and exfoliated in the lumen of the tubules especially in the testis of the treated groups with the high doses.

It has been cited, in other studies, that the combination of the fungicide Carbendazim and the related Benzimidazoles has an effect on testes and causes seminiferous tubular atrophy **(Hess et *al*., 2000, Lu et *al*., 2004)**. It could also reduce fertility and causes a dissociation of germ cells **(Cardone, 2012; Gawande et *al*., 2009)**, as well as the basement membrane that was severely ruptured especially for the animals treated with the high dose of MT, in addition to the presence of atrophy and vacuoles in affected seminiferous tubules. This result corresponds with many studies which confirmed that treatment of carbendazim caused a significant decrease in seminiferous tubule diameter **(Yu et *al*., 2009, Nakai et *al*., 2002, Gawande et *al*., 2009)**.

In this work, we hypothesized that MT could act as an endocrine disruptor by demonstrating that this fungicide altered testosterone levels in the plasma and in testis of adult male rabbits result in histopathological effects in the testis architecture.

Epididymis morphology was also altered by MT exposition, which was mainly demonstrated by dilation of seminiferous tubules especially in the groups treated with high doses of 100 mg/kg and 150 mg/kg. Our microphotographs sections of epididymis show some dilated tubules with irregular forms. In most specimens, there was coexistence of dilated tubules with those appearing normal. In addition, a few epididymis tubules were atrophic, considering these variable appearances of the tubules. On the other hand, the section of epididymis of rabbits treated with 150 mg/kg of MT was characterized by atrophy and great reduction in the seminiferous tubules lumen which were almost empty of the mass of spermatozoa.

In agreement with our findings about the effect of MT on male reproductive organs, it is consensual in literature that treatment with carbendazim causes testicular atrophy and decreases fertility **(Soni and Shrivastava, 2013).**

Concerning the result of hematologic parameters, it showed that red blood cells count decreased insignificantly ($P > 0.05$) below the control group as a consequence to the increase of MT dose administered to the rabbits, while it was significantly decreased ($p \leq 0.05$) in the group treated with a high dose of MT. However white blood cells count increased insignificantly in the group treated with the low dose of MT while it decreased

significantly in the groups treated with (100 and 150mg/kg), also hemoglobin and hematocrits content were decreasing with increasing dose of MT compared with the control.

Acute intoxication due to Carbendazim generally decrease RBC and haemoglobin content in the blood of rats **(Hayes, 1994)**. Red blood cells count decreased significantly in rats treated with 5 and 25 mg of Carbendazim **(Muthuviveganandavel et *al*., 2008).**

In other researches; WBC and lymphocyte counts decreased significantly at 10 mg/kg dose of carbendazim which suggests that this pesticide may possess an immune-suppressive potential in mammals **(Muthuviveganandavel et *al*., 2008).** Leucopenia in mammals following carbamate may be due to depression of leucopoiesis, alteration of cell membrane or disintegration of white blood cells. Moreover, a marked decline in haemoglobin concentration, hematocrit, and erythrocyte and leukocyte counts occurred at the highest zinc ethylene-bis-dithiocarbamate (zineb) after 90 days of treatment in rabbits **(Nebbia et *al*., 1995)**. **Zaahkouk et *al.* (2000)** witnessed a significant decrease of RBC, WBC, haemoglobin and hematocrit in rat blood after carbamate administration.

One of the molecular mechanisms of the toxicity of some pesticides seems to be lipid peroxidation; as a consequence, these compounds can disturb the biochemical and physiological functions of the RBC **(Akhgari et *al*., 2003)**. The susceptibility of RBC to oxidative damage is due to the presence of polyunsaturated fatty acid, haem, iron and oxygen, which may produce oxidative changes in RBC **(Kale et *al*., 1999).** Furthermore, the decrease in the number of red blood cells may indicate a disruption of erythropoiesis or an increase in the destruction of red blood cells **(Thomas et *al*., 1987)**.

Platelet also decreased with the increase of the MT dose and it was significant in the group treated with a high dose of MT. MCV and MCHC decreased insignificantly in all the treated groups, while MCH increased insignificantly in all the groups treated with MT compared with the control one.

The reduction in the blood parameters may be attributed to internal haemorrhage, possibly as a consequence of the toxic effect of carbamate on bone-marrow (**Capcarova et *al*., 2010)**. In return, in the review of literature, we found reports that Erythropenia in rats treated with carbamate may arise due to depression of erythropoiesis (**Reena et *al*., 1989; Sogorb et *al*., 2002; Capcarova et *al*.,2010)**.

Concerning thyroid function we recorded a significant decrease of T3 and T4 in plasma of the treated groups compared with the control. In the same context, later studies in rats showed that, Prochloraz decreased the concentration of thyroxin (T4) and thyroid stimulating hormone (TSH) in serum of exposed rats **(Vinggaard et al., 2002)** which has been more often studied. In one study Atrazine did affect the thyroid gland in female rats and dose- dependently decreased the serum T3 concentration **(Kornilovskaya et al.,1996)**. To our knowledge there is lack of data in literature concerning the Benzimidazols and its effect on thyroids function of rabbits.

Any environmental disturbance can be considered as potential source of stress, and theoretically it can be detected by changes in the plasma substrate concentrations. So that blood biochemical parameters including levels and activities of the organic and inorganic enzymes are generally believed to be good stress indicators and are influenced by various factors.

It is well reported in many research that the Carbamates cause significant changes in total serum lipids, glucose and protein levels in mammals **(Capcarova et *al.*, 2010).** We found insignificant ($P > 0.05$) increase of glucose level in the group treated with the low dose, while it increased significantly in the groups treated with 100 mg/kg and 150 mg/kg of MT. **Rodrigues et *al.* (1986)** found an increase in blood glucose level in rats treated with a single dose of 650 mg/kg of pesticide. Also the administration of pesticide Malathion increased plasma glucose concentrations (**Abdollahi et *al*., 2004, Capcarova et *al*., 2010).** On the contrary, **Sadeghi-Hashjin et *al.* (2008)** found a decrease of glucose level in mouse treated with Organo-phosphate pesticides**.** Moreover, the absorption of glucose was considerably reduced (35%) in another experiment with pesticide fed animals.

This finding could result from increased hepatic glycogen catabolism, as showed for other pesticides **(Kumar et *al*., 2009; Ksheerasagar et *al*., 2011)**. This metabolic change is similar to that observed in chronic undernutrition **(Emery et *al*., 2005)**. It is likely that increased plasma glucose levels in treated rabbits may be due to reduced glucose utilization and impaired tissue functions **(Chiali et *al*., 2013).**

Our results show a significant ($P \leq 0.01$ and $P \leq 0.01$) increase of cholesterol level in the groups treated with MT comparing with the control group. Other Investigation demonstrate

that the treatment with Carbendazim cause an increase in the level of cholesterol in rats **(Muthuviveganandavel et *al.*, 2008)** and similar results were obtained in the case of dogs **(Baron, 1991)**. Also a significant increase of cholesterol level after carbamate administration in rats was observed by **(Rai et *al.*, 2009).** This increase in cholesterol level may be a sign of liver damage **(Igbedioh et *al.*, 1992)** or the consequence of stimulation of catecholamine which stimulates lipolysis, and due to the increase of fatty acid production **(Dekundy et *al.*, 2007, Capcarova et *al.*, 2010).**

Our data show a significant increase in plasma triglyceride levels in rabbits treated with 100 mg/kg and 150 mg/Kg of MT while it insignificantly increased in the group treated with 50 mg/kg of MT compared to control rabbits. Our findings are in agreement with those found by **(Mallem et *al.*, 2006).** An increase in plasma triglycerides, glucose and cholesterol levels of male rabbits has been recorded after Dithiocarbamate treatment for a period of 5 weeks. Serum lipid concentration particularly cholesterol and triglycerides increased after 90 days of zinc Ethylene-bis-dithiocarbamate (Zineb) administration **(Nebbia et *al.*, 1995)** and an increased level of triglycerides in rats after carbamate administration for period of 1 week was found **(Rai et *al.*, 2009).**

We can suggest that this abnormality of increasing triglycerides could be a result of overproduction and/or decreased catabolism. As reported previously for other pesticides **(Chiali et *al.*, 2010)** an increase in liver lipid levels with a concomitant decrease in adipose tissue lipids was observed in rats exposed to Metribuzin.

These findings suggested an increased flux of plasma fatty acids from the mobilization of adipose tissue fat. This resulted in overproduction and secretion of plasma triglyceride – rich lipoproteins by the liver. Despite that, MT induced fatty liver in rats **(Ksheerasagar et *al.*,2011; Chiali et *al.*,2010).** This finding is agree with our results present in fig (8) shows macroscopic observation of fatty liver in treated rabbits compared with the control.

The results of our study propose that the administration of MT induces several metabolic changes. A biochemical investigation like plasma urea and uric acid are used to assess renal functions. Treatment of rabbits with MT at the used doses causes an increase in plasma urea and uric acid in all the treated groups, suggesting renal failure. Similar findings have been recorded by other pesticides **(Salem et *al.*, 2011; Chiali et *al.*, 2013)**. It is well known that urea is synthesized by hepatocytes from ammonia generated by amino acid catabolism from protein digestion in the intestines or from endogenous tissue proteins.

The rate of urea production depends on endogenous protein catabolism of organ protein contents (liver, muscle and adipose tissue). Increased protein catabolism was previously reported after pesticide exposure **(Ksheerasagar et *al.*, 2011)**. These findings are comparable to those obtained in food restriction which induced a protein catabolic state **(Emery, 2005).** In addition, we suggest that the increase in uric acid content in the serum is an indication of an increase in purine metabolism that remains targeted by these pesticides **(Muthuviveganandavel et *al.*, 2012).**

We also noticed a decrease in albumin level in the treated groups comparing with the control group, which may be a result of liver injury. Nevertheless, our results are in contrast with the ones obtained by **(Muthuviveganandavel et *al.*, 2012),** where albumin was increased in the treated rats with (0.48, 0.96, 2.4 and 4.8 mg/kg bw) of carbandazim for 24h.

In regard to the histology of liver and kidney, many investigations reported that Carbendazim causes hepatic toxicity **(Lu et *al.*, 2004)**. Earlier results also revealed an increase in liver weight after MBC exposure **(Sherman, 1972)**.

In the present study, the morphology of the liver seemed to be mostly affected by MT treatment. The changes were large sized; cytoplasmic vacuolization, fat vacuoles, disruption of hepatic architecture, necrosis and dilated congested blood vessels. Accordingly, Benzimidazole fungicides are known to induce various histopathological changes in the liver tissues. Such as hemorrhage, inflammatory cell infiltration, tissue damage and necrosis **(Soni and Shrivastava, 2013)**, also pesticides have been found to affect the cytochrome p450 system or the mitochondrial membrane transport system of hepatocytes **(Gokcimen et *al.*, 2007)**. Recent studies also revealed that MBC exposures induce histopathological changes in the liver and kidneys **(Soni and Shrivastava, 2013)**.

On the other hand, the histological examination of kidneys of the control rabbit revealed normal histological features. On the contrary, the administration of MT causes much histological damage to the renal cortex. Examination of the kidney sections of animals after treatment with MT for 4 weeks showed that renal blood vessels were congested. Most of renal tubules were damaged and lost their characteristics. This findings is in agreement with the other study which showed that carbandazim exposure induces histopathological

changes in rats' kidneys after treatment of 30 days with daily dose of 33 mg and 100 mg/bw **(Soni and Shrivastava, 2013).**

4.2. Impact of Thiram

Thiram is fungicide used to protect plants from fungal diseases but It is also used as an animal repellent to protect fruit trees and ornamentals from damage by rabbits, rodents and deer. LD50 of Thiram in rabbits by orally rout is 210 mg/kg but in the present work we recorded a rate of mortality of 100% in the group treated with the high dose 80 mg/kg/bw. This fatal effect may be is result of hemorrhage induced by Thiram. While the Macroscopic examinations of the lung of treated animals and one dead rabbits of the groups treated with the high dose of Thiram revealed subpleural and intraparenchymal hemorrhagic spots (fig 27). Moreover the environmental conditions and species of rabbits have a major role in the sensibility of animals to the toxic elements.

Concerning the effect of Thiram on fertility, earlier studies reported that Dithiocarbamate has destructive effects on different levels especially in male reproductive system; it has a marked spermicidal activity **(Rice, 1964).**

The data show a decrease of testes and epididymis weights of the treated group with Thiram compared with the control group. Similarly, **Slimani et *al.* (2015)** found that the treatment of male pigeons by 1 g/l of Maneb during six weeks induced a decrease of testes weight.

Sperm concentration or density is referred to the count of sperms per milliliter of semen. In most cases of semen studies, sperm concentration is the most common factor to be determined.

The results in our work indicate a low caudal epididymal sperm density; may be due to alteration in androgen metabolism, in which the physiological and biochemical integrity of epididymis are dependent on androgens **(Brooks, 1979)**. It was reported that the 80% negative fertility test may be attributed to the lack of forward progression and reduction in density of spermatozoa and altered biochemical milieu of cauda epididymis **(Joshi et *al.*, 2003).** Similar study conducted by **(Mallem et *al.*, 2007)** found that rabbit's receiving Maneb had decreased testes weight with a decrease in sperm concentration and motility, disruption of spermatogenesis reflected by the significant decrease in the number of spermatogenic cells and sperms.

Our data show a significant decrease of spermatozoa motility in the treated groups compared with the control so many hypotheses have been proposed to explain the effect of fungicide on sperm motility.

Sperm motility depends on the integrity of the mid-piece and tail for producing energy to move. The main source of energy for spermatozoa is Adenosine Triphosphate (ATP). In process of ATP synthesis, protein phosphatases and protein kinases play significant roles. Therefore, any factor interfering the assembling of tail structure protein components and/or modifying the concentration/function of ATP synthesis can lead to decreased sperm motility **(Mehrpour et *al*., 2014; Perry et *al*., 2011).** In a study conducted by **Lifeng et *al*. (2006)**, it was shown that sperm motility and speed could indirectly reflect it's fertilizing ability. Other study found that sperm motility could be affected by some reproductive toxicants such as fungicides. Therefore, measurement of sperm motility is a more sensitive method to study male reproductive toxicity**.** It is thought that fungicides exposure could reduce seminal volume and sperm motility, increase seminal PH, and increase abnormal sperm morphology particularly at head level by affecting epididymis, seminal vesicle, and prostate functions **(Yucra et *al*., 2006; Choudhary et *al*., 2008).**

In this study plasma testosterone levels significantly decreased in Thiram-treated rabbits compared with the control group. The lower plasma testosterone level in Thiram treated rabbits could be attributed to impaired Leydig cells, similar study by **Ilbey et *al* (2009)** was found that Pyrrolidine Dithiocarbamate reduce plasma testosterone in albino rats.

Indeed Dithiocarbamate like endocrine disrupters has the ability to affect testosterone production directly or by influencing the control of gonadotropin production, since the male reproductive endocrine system involves components from the hypothalamus and pituitary as well as the testes. Thus, they influence the synthesis or release of FSH, LH, including the chemicals that interfere with hormone receptor synthesis or function **(Campion, 2012).**

In this study, administration of Thiram reduced weights of testes and epididymis compared with control group, which show consistency with previous reports which claimed that Dithiocarbamate decreases reproductive organ weights. Practically the reductions in testis weight were due to marked parenchymal atrophy, which were appear very well in our results of microphotographs sections of testes and epididymis, similarly the same results were found by **(Mallem et *al*, 2007).**

In regards to the results of our histological study of reproductive organs, the microphotographs of testis and epididymis showed a severe degeneration, necrosis and reductions in seminiferous tubules of testes of the treated animals. Furthermore the observation of epididymis sections indicates a decrease in the volume of spermatozoa in epididymis tubules of rabbits treated with Thiram. On the other hand the microphotographs of the control sections showed a normal histology of testes and epididymis, consequently the seminiferous tubules has all the successive stages of spermatogenesis and the lumen of epididymal tubules contain a significant volume of mature spermatozoa's.

On the whole the effects of this agent on the testis might be due to its specific toxic impacts on the target organ **(Ilbey et *al*., 2009).** Concerning the results of hematological study, we observed that red blood counts decreased insignificantly ($P > 0.05$) for the treated group by 20 mg/kg and decreased significantly for the group treated with 30 mg/kg of Thiram as compared to the control group. Consequently, hemoglobin content was increased insignificantly in the treated groups with Thiram, it can be the result of red blood destruction (hemolysis) influenced by Thiram.

Hematology is a valuable tool for assessing the injuries caused by Carbamate. Then the decrease in the red blood parameters may be attributed to internal haemorrhage, possibly as a consequence of the toxic effect of Carbamate on bone-marrow, so erythropenia in rats treated with Carbamate may arise due to depression of erythropoiesis **(Zaahkouk et *al*., 2000).**

On the other hand, white blood cells count also increased in the treated groups with Thiram, which would suggest that this fungicide may stimulate white blood cells, because WBC combats against any xenobiotic agent introduced into the blood **(Muthuviveganandavel et *al*., 2008).**

The results obtained in the present study from the impact of the used fungicide on Thyroid activity revealed a decrease in plasma Tri-iodothyronine (T3) and Thyroxin (T4) concentration in treated animals with Thiram compared with the control animals.

Thyroid hormones are major mediators of basal metabolic rates and together with other neuronal and endocrine components; they contribute to systemic coordination of energy **(Kim, 2008)**. Other work has shown that Dithiocarbamates affect the secretion of TSH

(Thyroid Stimulating Hormone) in first place **(Wayland, 1991)**. Thus it disrupts the thyroid gland function and decreased the secretion of thyroids hormones **(Bhaskar and Mohanty, 2014)**. This explains hyperlipidemia which means an increase of the plasma concentration of cholesterol and triglyceride and hyperglycemia as a consequence of metabolism imbalance.

Hepatic fat metabolism is affected in various ways by different types of Dithiocarbamates such as Nabam, Zineb and Thiram. In our experiment, Thiram increased Triglyceride Cholesterol and Glucose level, perhaps as a result of fatty acid mobilization as well as the hypo-function of the thyroids.

The increase of cholesterol level in treated groups compared with the control is similar with other results obtained in the case of dog **(Baron, 1991).** Significant increase of cholesterol level after administration of Carbamates (Cartap and carbofuran) for one week in rats was observed **(Rai et *al.*, 2009).** It is important to recall that the increase in Cholesterol level is a sign of liver damage **(Igbedioh et *al.*, 1992).** These results are in agreement with other investigations which found that an increase in plasma Triglycerides, Glucose and Cholesterol levels of male rabbits has been recorded after Dithiocarbamate treatment for a period of 5 weeks **(Mallem et *al.*, 2006).**

Data show an increase in urea level in the treated rabbits with Thiram compared with the controls. Urea is the principal end product of protein catabolism. Enhanced protein catabolism and accelerated amino acid deamination for gluconeogenesis is probably an acceptable postulate to interpret the elevated level of urea. This elevation in urea might be also due to the destruction of red blood cells during the treatment. The presence of some toxic compounds may increase blood urea and decrease plasma protein **(Ashour, 1999; Zaahkook et *al.*, 2009).** Furthermore, serum uric acid levels exhibited significant increases in treated rabbits. This may be due to high degradation of purines, or caused by an inability to excrete by the urinary system **(Wolf et *al.*, 1972; Abdel Aziz and Zabut, 2014).** Elevation of blood urea and uric acid are good indicators for kidney diseases **(Abdel Aziz and Zabut, 2014).** Also we noticed a decrease in Albumin level in treated groups compared with the control, can result of liver damage.

Moreover, investigation revealed that the chronic toxicity profile for Thiram indicated that the liver, and urinary system are also the target organs for this fungicide **(EPA, 2004),** Results obtained in this work showed that Thiram induces an increase in the liver weight of

rabbits given fungicide. Similar results noted an increase in the weight of liver in pigeons exposed to Maneb **(Slimani et *al*., 2015).**

In addition our photomicrographs sections of liver demonstrate many histopathological changes in liver of treated animals, revealed infiltration and necrosis of hepatocytes. The effect of fungicides on mammalian tissues was investigated by many searchers, **Lamfon et *al*. (2011)** reported that Metalaxyl induced hepatotoxicity in albino rats. **Szepvolgyi et *al*. (1989)** reported that when male and female rats were exposed to Mancozeb, the liver showes a necrosis and congestion of blood vessels. also earlier study by (**Özbay et *al*., 1991)** reported that exposing mice to the fungicides, Maneb and Zineb causes blood congestion and mononuclear inflammatory cell infiltrations in the liver **(Capkin et *al*., 2010; Gul et *al*., 2012; Rasgele et *al*.,2015).**

Concerning the Sections of the control rabbit's kidneys are demonstrate a normal renal histo-architecture of the kidney include glomerulus and surrounding tubules. However the kidneys of the treated rabbits by Thiram shows marked necrobiotic changes, a marked necrosis of tubular cells, atrophy of the glomerulus, and areas of interstitial infiltration of round cells were found as compared to the normal histological examination of renal tissue in the control rabbits. These results go in the same direction of the one obtained by **Abd-Elhady and Abou-Elghar (2013)** who demonstrated that the administration of another pesticide Abamectin by 10 mg/kg once a week for 210 days and 30 mg/kg three times a week for 30 days induces histopathological changes in the liver and kidneys of treated rats**. Szepvolgyi et *al*. (1989)** also demonstrated that pesticides cause a tubular dilation, necrosis and congestion of blood vessels in kidney of treated animals by Dithane.

Conclusion & Perspectives

5. CONCLUSION

Our research project aims to assess the toxic potential of two classes of fungicides, Benzimidazol (MT) and Dithiocarbamate (Thiram) for agricultural use, to carry out our research project, fertility, hematological, biochemical, hormonal parameters and histological sections were measured in control rabbits and treated with fungicides for a period of 30 days by MT and 15 days by Thiram. The results can be summarized in following points:

From the preceding discussion, it is evident that fungicides alter male fertility by affecting semen quality, proof of this: a decrease in the number, motility, speed and vitality of sperm of the treated groups with histopathological changes of the reproductive organ (testes and epididymis) and a decrease in the testosterone level, which confirms the anti-androgenic effect of these pollutants.

In regard to the hematological function: The administration of fungicides causes a disruption of hematological parameter in the treated groups by fungicides compared to control.

Biochemically, the toxicity of two fungicides were manifested by a metabolic imbalance mainly characterized by hyperglycemia, hyperlipidemia, increase of cholesterol and change in renal and liver function tests.

The widely used fungicides MT and Thiram have been shown to causes alteration in thyroids function.

As well histological sections performed on liver, kidney, testis and epididymis fragments, also confirmed the adverse effect of fungicides on function and histology of organs.

Whereas the high doses of Thiram was fatal to all treated rabbit within 2 weeks

7. PERSPECTIVES

We look in the next work to achieve:

- ✓ The effect of these fungicides in both sexes, in particular in male and female sexual activity and pregnancy, using other cellular and molecular technics to better understand these effects at molecular level.
- ✓ See the long-term effects (at least two generations) of this type of stress, and whether these effects disappear by time or develop other diseases with therapeutic study using medicinal plants.
- ✓ Determination of oxidative stress parameters in different organs (liver, kidneys, testes and epididymis).

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Evaluation of Methyl Thiophanate Toxicity on Fertility Andhistology of Testis And Epidydim In Male Rabbit

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ABSTRACT

The purpose of this study is to determine the toxic effects of fungicide Methyl Thiophanate (MT) on fertility and testes histopathology in male domestic rabbits. Methyl Thiophanate is a carbamate fungicide of Benzimidazol family. Mature rabbits were divided into four groups. The first is the control one, while the other three groups are orally treated by Methyl Thiophanate (MT) with daily doses of 50,100 and 150 mg/kg/day of body weight for four weeks. On day 31 of the experiment, the animals were weighed and sacrificed. The testosterone level was measured. Biological study of spermatozoa was performed,and the histology study was carried out. The obtained results show that MT altered fertility in male rabbit by reduction inquantity and qualityof spermatozoa (concentration motility, speed and viability).A reduction in testosterone level and atrophy in testes and epididymis were observedin the treated animals with MT as compared to the control group.

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INTRODUCTION

Pesticides are contemplated to be the worldwide contaminants and shown to exert various toxicological effects in humans and animals [1,2,3]. There are a number of possible ways in which humans can be exposed and toxic effects of these pesticides may haveconsequences to food consumers, production workers, formulators, farmers and other applicators. Fungicides are extensively used against a wide range of fungal diseases of many field crops, fruits and ornamentals. On the other hand, some of fungicides have showntoxicity to humans, animals, and useful plants, in addition to its persistence (long life) in the environment. Moreover, these chemicals were shown to be present in fruit products prepared for human consumption [4].

Recently, a possible role of fungicides in the decrease of sperm quality and in otherdisorders of the male reproductive tract has been reported [5].

Methyl Thiophanate (1, 2-Bis (3-(methoxycarbonyl)-2-thioureido)benzene) is a systemic broad-spectrum fungicide controlling various fungal pathogens. The LD 50 of Methyl Thiophanate in the male rabbit by oral route is 2270 mg/kg body weight. It is used as a preservative in paint, textile, papermaking, leather industry and warehousing practices, as well as a fruitpreservative of [6,7]. Methyl Thiophanate is well absorbed (80–85%) after oral exposure and is subsequently metabolized into many compounds within the organisms. The main metabolite is methyl 2-benzimidazolyl carbamate (carbendazim) [8,9]. Carbendazim is poorly catabolized and remains in tissues such as gonads, liver, adrenals, adipose tissue, skin and other organs [10]. Itisa well-recognized testicular toxicant [11,12,13,14,15].

Evidences available support the deleterious effects of Methyl Thiophanate and itsmetabolite carbendazim on various aspects of male reproduction in hamsters, mice, rats and humans.The effects include the decrease ofaverage testes weight,average seminiferous tubular diameter [12], total sperm count, motility, increasedincidence of sperm abnormalities [16] and disruption of microtubule formation [17]. Carbendazim, an inhibitor of microtubule synthesis, directly alters testicular function via germ cell depletion, alterations of Sertoli and Leydig cellular functions [18]. In addition, it has been reported that lesions in the male tract cause blockage which may induce permanent testicular damage and a decrease in sperm production exposed to benomyl and carbendazim in adult rats [13].

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Hence, the present study was undertaken to investigate the reprotoxic effect of fungicide Methyl Thiophanate on fertility and the histology of testis and epididymis in adult male rabbit.

MATERIAL AND METHODS

Animals and treatment:

Mature male rabbits*Cuniculus lepus* were obtained from the region of Annaba(eastern Algeria). Those Animals were housed in special cages and were maintained in naturalconditions of temperature and relative humidity. Animals had access *ad libitum* to pure water and food.

Theywere divided into 4 groups,each contained 6 rabbits. Group 1 served as a control one. Groups 2, 3 and 4 were treated orally with Methyl Thiophanate dissolved in distilled water at a dose of 50, 100 and 150 mg/kg of body weight respectively for 31 days.After sacrifice by decapitation, blood and semen werecollected and the testes of each animal ofthe different groups were removed and weighed.

Biological study of spermatozoa:

Immediately after the end of the treatmentperiod, a small opening at the epididymis was carried outto obtain a drop of sperm of about 1µl, then 49 µl of 0.9% NaCl was added to evaluate the concentration, speed, motility and vitality of spermatozoa as described by OMS 1993method[19].

Testosterone assay:

After collecting blood, samples were centrifuged with 5000 tr/min to isolate serum, The serum testosterone is proportioned by the conventional method ELISA using commercial kit. Measurement is done using a reader ELISA TECAN Magellan provided with data processing software which calculates the range standard automatically and the level of the testosterone to the used unit directly.

Histology examination:

Testes and epididymis samples were fixed in 10% formol and dehydrated in 70-100% ethanol series. They were then embedded in the paraffin baths at 58℃ for paraffin inclusion. Sections of 4-6 µm were prepared from paraffin blocks using a rotary microtome. These sections were then stained with Hematoxylin-Eosin (H-E) according to the criteria of Martoja 1993method**[20].** Sections werephotographed using a photomicroscope.

Statistical analysis:

All results were expressed as mean ± SE and analyzed using *t*-Student test with the Minitab program (version15) comparing each treated group with the control. The significance level of $p < 0.05$ was considered.

Results:

Weight of testes and epididymis:

The table 1 revealed a highly ($P<0.01$) and very highly significant ($P<0.001$) difference in the weight of testis and epididymis of animals treated with MT as compared to control.

Table 1: weight of the testes and epididymis in experimental rabbit treated with MT compared to the control group.

Weight (g)	G1: Control	G2: 50mg/kg	G3: 100mg/kg	G4: 150mg/kg
Testes	2.27± 0.20	1.22± 0.19***	0.99± 0.20***	1.09 ±0.07***
Epididymis	0.5± 0.11	0.28± 0.06**	0.23± 0.08**	0.22 ±0.05***

Values are mean ± SE (n=6) P<0.05*, P<0. 01**,P<0.001***compared tocontrols

Semen study:

The study of Epididymal sperm shows that the sperm count in(Fig1) and sperm motilityin (Fig 2) havesignificantly decreased ($P<0.001$) in the treated animals as compared to the control group. The Fig 3 andFig 4 also indicate a decrease in the speed and the percentage of alive spermatozoa with an increase in the dead spermatozoa in the experimental groups as compared to the control one.

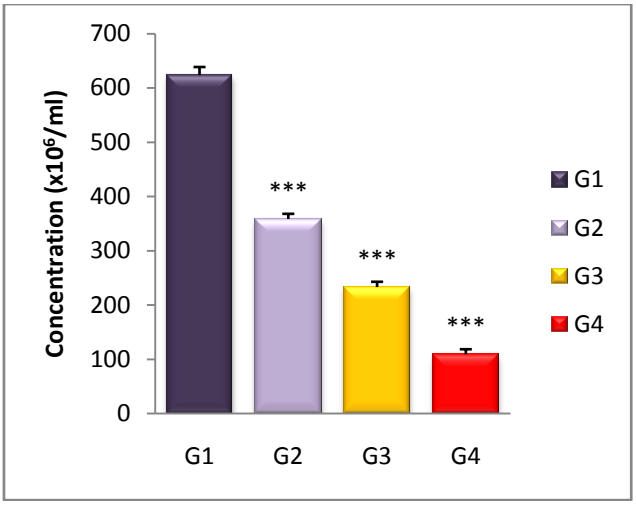

Fig. 1: Epididymal sperm concentration of control and experimental groups (x10^6/ml).Values are mean ±SE(n=6), P<0.001*** compared to control.

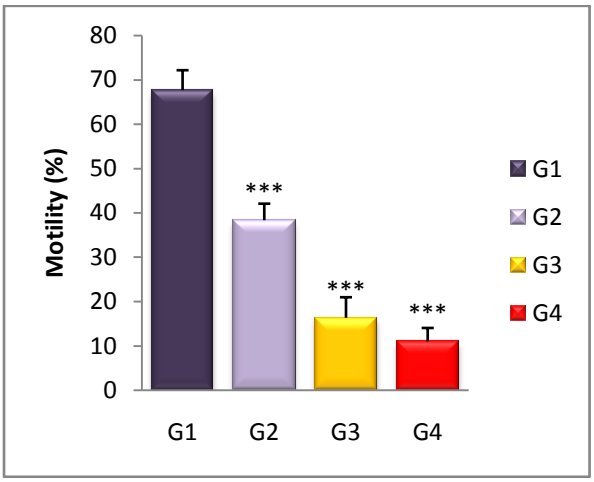

Fig. 2: Epididymal spermmotility of control and experimental groups(%).Values are mean±SE (n=6), P<0.001*** compared to control

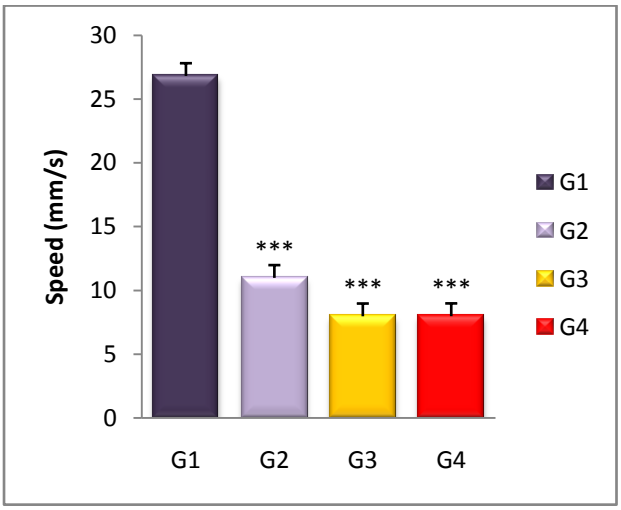

Fig. 4: Epididymal sperm viability of control and experimental groups**) (%),** Valuesaremean±SE (n=6), P<0.001*** compared to control.

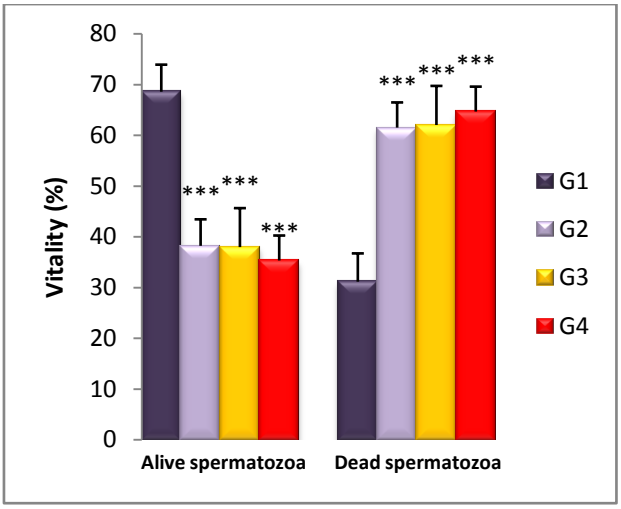

Fig. 3: Epididymal spermspeed of control and experimental groups (mm/s), Values are mean±SE (n=6), P<0.001*** compared to control.

Serum testosterone level:

Change in testosterone level in all groups of rabbits is reported in the Fig 5. The results revealed that the serum testosterone levels were significantly reduced (P<0.05) in MT treated rabbits when compared to the control animals.

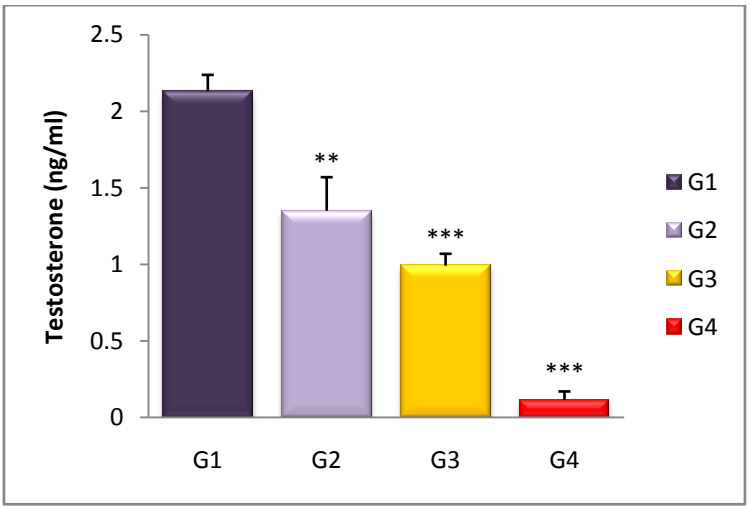

Fig. 5: Serum testosterone level changes(ng/ml) of control and treated groups.Values are mean±SE (n=6), P<0.01**, P<0.001*** compared to control

Histology study:

The microscopic examination of testis of control rabbits (G1) has showna normal spermatogenesiscells in different phases and a lot of spermatozoa in the somniferous tubules reported in Fig 6.

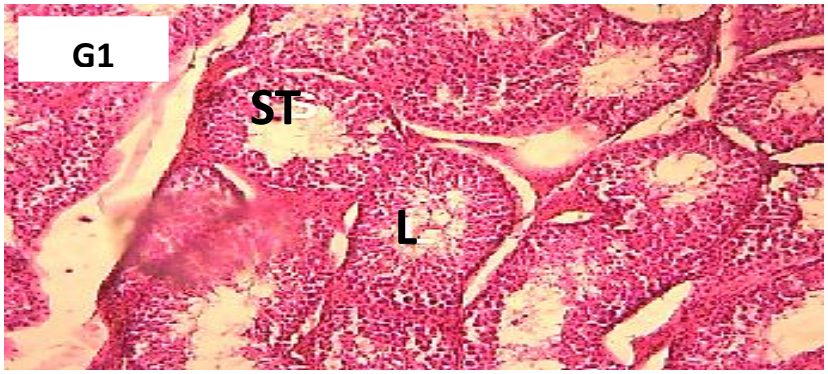

Fig. 6: Rabbit's testis section of the control group.(Hemtoxylen and eosin X120).

The testis of all groups showthat the membrane of the somniferous tubules is full determined, and all stages of spermatogenesareclear. Lumen (L) of each tubule is almost occupied by the mass of the mature spermatozoa's. (ST:seminiferous Tubules).

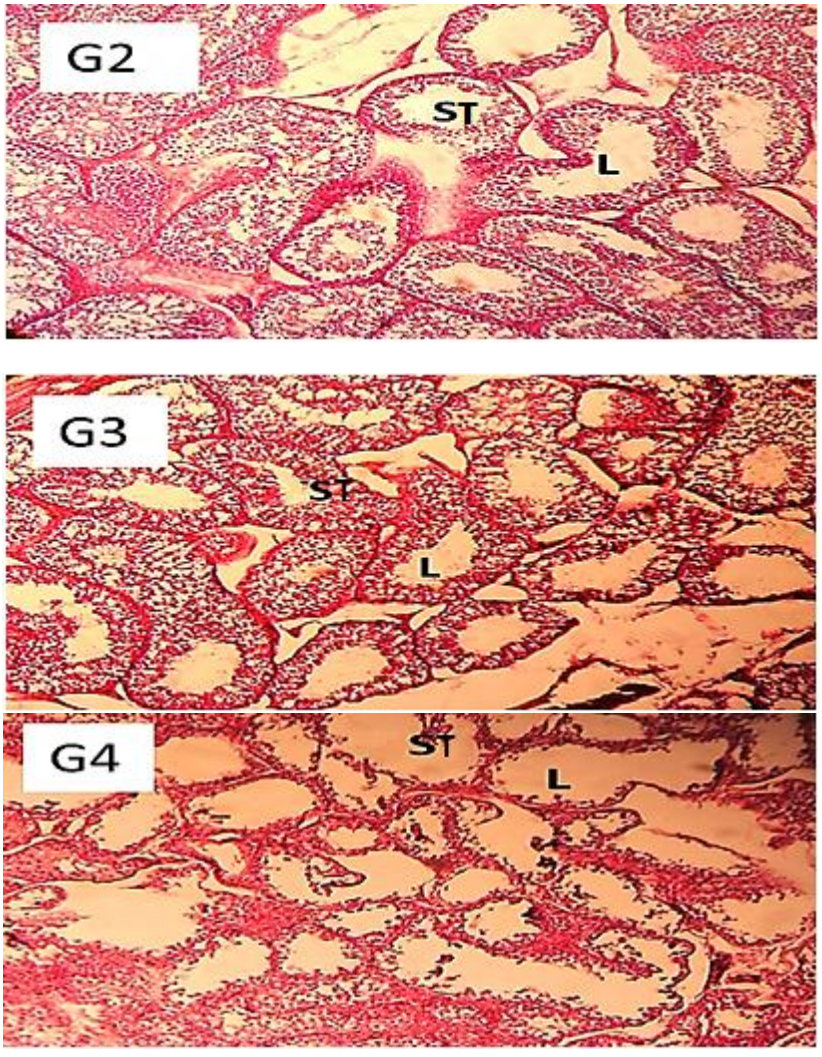

Fig. 7: Rabbits'testis sections of treated groups. (Hemtoxylen and eosin, X120).

The sections of testis intreated groups haveshowna decrease in the number of spermatozoa inseminiferoustubules (G2,G3)and they aredeformed in testis section of the high dose (testis of 5 animals of G4) where the tubules membrane is broken and the lumen (L) ofseminiferoustubules arealmost empty with large disturbance stages of spermatogenesis.

Light microscopic examinations revealed that the animals which weregiven Methyl Thiophanate for 4 weeks (G2, G3 and G4) exhibited a distinct histological difference with the control group. Thetestissection of the treated animals indicates a considerable decrease of spermatozoa in lumen of seminiferous tubules.

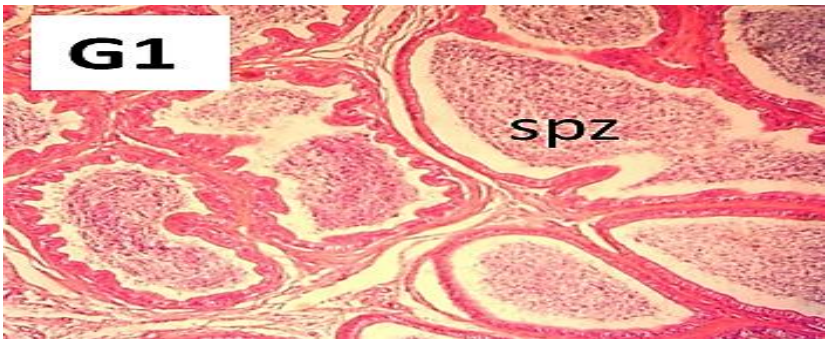

Fig. 8: Rabbit's epididymis section of the control group (G1). (Hemtoxylen and eosin X120).

The epididymis section of the control group has shownthat the Lumenof the epididymal tubules is full of spermatozoa (SPZ)and the tubules epitheliums are full determined.

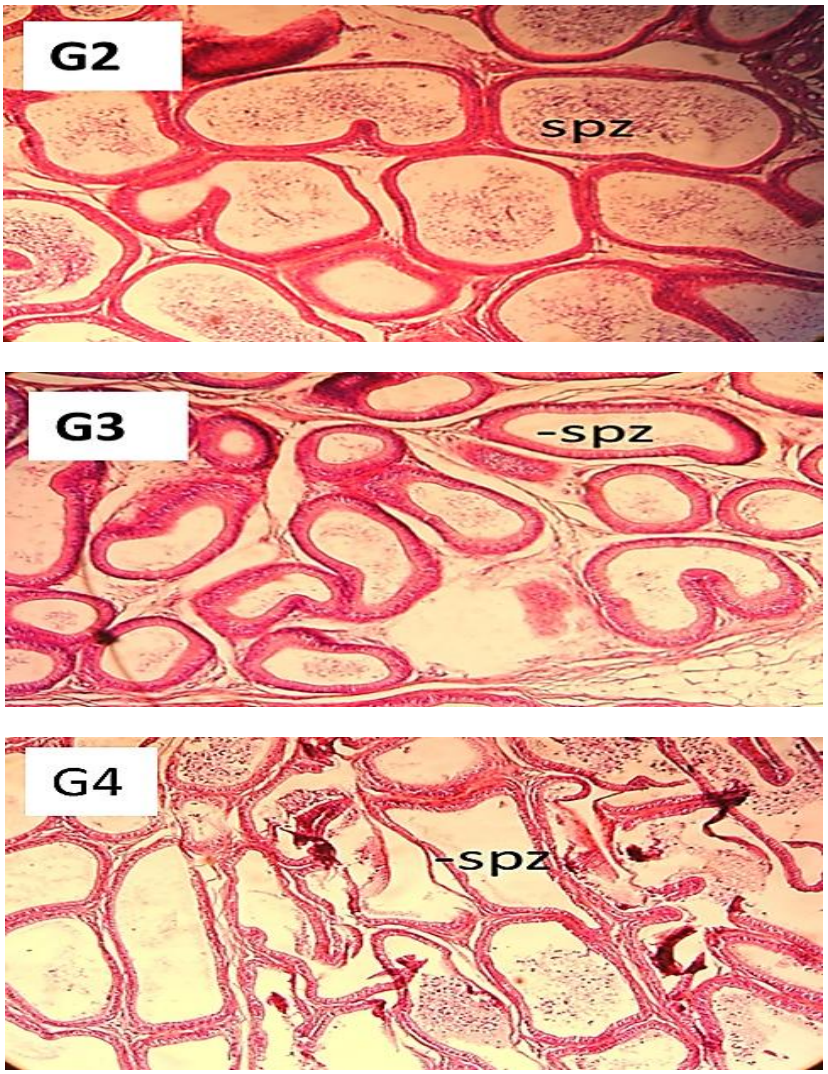

Fig. 9: Rabbit's epididymis sections of the treated groups, (Hemtoxylen and eosin, X120)

Theepididymis sections in the treated groups have showna considerable decreaseof spermatozoa's (SPZ) volume observed in epidydimis tubules of (G2 and G3) and is almost empty in high dose epididymis section (G4).

Discussion:

The present study was carried out to determine the potential adverse effects of MT on spermatogenesis and fertility of male rabbits.

The first result from this study is that the weight of testis and epidydimis of the treated rabbits decreased considerably as compared to control group. It is reported that exposure to MethylThiophanate, a recognized reproductive toxicant, can negatively affect the human reproductive system. Effects on the male reproductive system can include suchanaltered sexual behavior, altered fertility and problems with sperm shape or count [21].

The decrease of the testis weight resultedfromthe susceptible effectsof MT on histological constitution and morphology of Sertoli Cells. In the other hand, it is admitted that the gonads are the target bodies of pesticides [22]. A similar study has shownthe same observations on the rats treated with the fungicide Carbendazim [23].

The Reduction in serum testosterone level in MT treated rabbits, maybe a result of a cumulative effect of decreased Leydig cell number. Therefore, they decreased testosterone synthesis and secretion. This decline in testosterone could probably be due to the diminished responsiveness of Leydig cells to LH and/or the direct inhibition of testicular steroidogenesis [24] It is well known that LH is the prime regulator of testosterone production by the Leydig cells [25]. The Carbendazim is one of the important metabolite of MT. Several papers have reported that treatment of experimental animals with the fungicide Carbendazime (MBC) brings about disruption of spermatogenesis [13,26]. Then, Thiophanate Methyl can affect the hypothalamic and hypophyseal hormones [27] by disturbing the secretion of GnRH which stimulates adeno-hypophysis to secrete LH and FSH by modifyingthe receiving sites affecting testosterone secretion [28, 29]. This explains the decrease of the speed and motility of the spermatozoa in the treated groups, especially those treated with high doses as compared to the control. A similar study with carbendazim which administered to Wistar male ratsreported that the motility of spermatozoa is due to the flagellar beat which inturn is dependent on the microtubular apparatus of the flagellum, as MBC is a microtubular poison.MBC, through testicular and epididymal routes, would affect the sperm flagellar microtubules, which leads tosperm motilityimpairing [16].

The loss of cauda epididymal sperm motility in the treated rabbits is probably related to a change in sperm membrane properties [2], hasreported thepossibilitythat the carbendazimmakes access into the epididymis and alters it in respect of function towards initiation of sperm motility [16]. A similar study on the rats indicates that the used fungicide MT caused dysfunction inthe spermatogenesis and Sertoli cells [30]which is necessary to the maturity of seminiferous tubules, throughout its role of nutrition of spermatozoa and this alters a dysfunction of the sperm count [31,32,33].

This is further supported by a reduction in sperm viability, where the percentage of the alive sperm was declined. Available reports haveshownthat most of fungicides like mancozeb are inhibitors of spermatogenesis at gonadal level by affecting hormone production [34]. The histopathological changes are observed in epididymis and especially in the testes. Treating animals with MT caused significant decrease in diameters and germinal epithelial heights of the seminiferous tubules. Histological results revealed that the spermatogenic cells were degenerated and exfoliated in the lumen of the tubules [35]. It is reported that the combination of the fungicide Carbendazim and the related benzimidazoles, have an effect on testicular and seminiferous tubular atrophy [13,14]. It could also reducefertility and causesa dissociationof germ cells [9,13,35]. The sections of testes and epididymis showed that MT decrease sperm directly in epididymis.The treatment with MT is increasingdead sperm, and histological examination revealed atrophy of the seminiferous epithelium and moderate impairment of spermatogenesis and dissociation of germ cells,as well asthe basement membrane which was severe in the high dose. This result corresponds tomany investigators, they confirmed that treatmentof carbendazimcauseda significant decrease in seminiferous tubule diameter, and epithelial height. Histopathological results showed degeneration and decrease of spermatogenic cells, atrophic and reduced testis and epididymis [15,17,36].

Conclusion:

It is concludedthat the treatment of Methyl Thiophanate is toxic to spermatozoa in the caudaepididymis; it causesa decrease in the sperm counts, motility and viability of spermatozoa. This paper showsclearly that theMT treatment for 4 weeks caninducea decrease in the quality of the sperm with histopathological changes in the testes.

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