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2 **PROTECTIVE EFFECTS OF ALLIUM CEPA ON TESTIS OF**
3 **RATS EXPOSED TO GLYPHOSATE**

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7

8 **Abstract:**

9 **Background:** Glyphosate [N-(phosphonomethyl)glycine] is one of the most widely used
10 organophosphorus herbicides. Allium cepa (AcE), popularly known as onion, has been reported
11 to have an antioxidant properties in both rats and human.

12 **The aim of this study is** to investigate the histological, and immunohistochemical effects of
13 Glyphosate (GP) as an Organophosphrous compound (OPC) on rat testis and to assess the
14 protective effect of Allium cepa on testis of rats exposed to glyphosate.

15 **Materials and methods:** This study included 30 adult male Albino rats divided into
16 3groups. Group I (Control group): Each rat received distilled water(0.2 ml/day). Group II: Each
17 rat received glyphosate at a dose of 125 mg /kg, body weight. Group III: Each rat was given
18 Allium cepa (AcE) 1 ml/100 g BW two hours before administration of GP at a dose of 125 mg
19 /kg body weight. All the drugs as well as distilled water were given daily by oral gavages for
20 30days . The sections of testis were stained with Hematoxylin-Eosin (HE) and Masson's 20
21 Trichrome stains .Also Immunohistochemical study was done to detectBCL2. Morphometric 21
22 study: The mean area percentage of collagen fiber deposition and BCL2 immuno-expression 22
23 was quantified in five images from five non-overlapping fields of each rat . The data were 23
24 collected from the experiment , recorded and analyzed using IBM SPSS Statistics software .

25 **RESULTS:** The study had demonstrated that glyphosate caused a degeneration of all layers of
26 the germ cells, congestion of the blood vessels, and increased of the collagen fibers in the
27 capsule . Immunohistochemical results showed a decrease in the expression of the
28 antiapoptotic protein(Bcl2) . Allium cepa administration partially ameliorated the degeneration
29 effect of glyphosate on the seminiferous tubules.

30 **Conclusion:** Allium Cepa protects the testis from the toxic effect of glyphosate

1 **Keywords:**

2 Glyphosate – Allium Cepa- antiapoptotic protein(Bcl2)- Immunohistochemistry.

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6 **Introduction:**

7 Glyphosate [N-(phosphonomethyl)glycine] is one of the most widely used organophosphorus
8 herbicides [Cerdeira et al.,2007]. Glyphosate is a broad-spectrum herbicide effective against
9 weeds and represents approximately 30% of all herbicides used in agriculture as well as garden
10 maintenance including home use [Zahran et al.,2005]. It is the active ingredient of more than
11 700 different broad spectrum herbicides [Abarikwu et al., 2015].

12 Although glyphosate is considered a low-toxic herbicide, recent studies have revealed toxic
13 effects resulting from even low-dose commercial formulations [Benachour et al. 2007]. The
14 herbicide glyphosate is considered as a potential endocrine chemical disruptor which interferes
15 with the production, release, transport, metabolism, binding, action and elimination of natural
16 hormones responsible for the regulation of developmental processes [Kavlock et al. ,1996]

17 Organophosphorous compounds partially exert their pathological impacts via promotion
18 of oxidative stress in reproductive tissue [Milatovic et al.,2006]. Accordingly, OP agents
19 increase oxidants by disrupting enzymatic and/or non-enzymatic antioxidant defenses as well
20 as enhancing high energy consumption coupled with inhibition of oxidative phosphorylation
21 [Razi et al.,2012]. In addition, oxidative stress may cause degenerative alterations in sperm
22 cells due to the high levels of polyunsaturated fatty acids (PUFA) in their plasma membrane
23 [Agarwal and Allamaneni 2006]. Imbalanced generation of oxidants affects the integrity of the
24 sperm's DNA by causing elevated frequencies of single strand DNA (SS-DNA) and double-strand
25 DNA (DS-DNA) breaks [Fraga et al.,1996].

26 Allium cepa (AcE), popularly known as onion, has been reported to have antioxidant properties
27 in both rats and human [Cavagnaro et al.,2007 and Khaki et al., 2009] as it Contains
28 antioxidants such as selenium, glutathione, vitamins A, B, and C, and flavonoids such as
29 quercetin and isorhamnetin. [Kumar et al 2016].

30 The aim of this study is to investigate the effect of Glyphosate (GP) as an Organophosphorous
31 compound on rat testis and to assess the protective effect of Allium cepa on testis of rats
32 exposed to glyphosate.

1 **Materials and methods:**

2 **A- Animals:**

3 Thirty adult male albino rats aged 8 weeks old, were used in this work. Their weight
4 ranged from 200-250gm each .The rats were obtained from the Animal House of the Faculty of
5 Veterinary Medicine, Benha University, Egypt. They were housed in a plastic cages at room
6 temperature with 12 hours light and dark cycle. They were fed balanced diet consisting of milk,
7 vegetables and bread. All rats were kept under the same circumstances throughout the
8 experiment.

9 **B- Drugs:**

10 **1- Preparation of Allium cepa Extract:**

11 AcE was obtained from fresh Allium cepa (common onion) bulbs that were rinsed thoroughly in
12 distilled water, air-dried, and 200 grams were then blended. The resulting paste was allowed to
13 stand for 24 hours. Then Juice was filtrated and squeezed out of it using a tight sieve. The
14 filtrate was prepared on weekly basis following the same procedure and kept at 4°C to prevent
15 it from losing its potency [Azu etal.,2007].

16 **2- Glyphosate:**

17 We obtain it from Sigma pharmaceutical CO., Egypt as a white powder which was
18 dissolved in distilled water and given at a dose of 125mg/KG body weight

19 **Experimental desigen:**

20 The rats were divided into 3 groups. Each group consisted of 10 rats.

21 Group I (Control group): Each rat received distilled water(0.2 ml/day) by oral gavage, once a day
22 for 30 days.

23 Group II: Each rat received GP at a dose of 125 mg /kg, body weight, by oral gavage, once a day
24 for 30days.

25 Group III: Each rat was given 1 ml of Allium cepa (AcE) /100 g BW two hours before the
26 administration of GP at a dose of 125 mg /kg body weight by oral gavage for 30days .

27 **Histopathological analyses:**

28 After 30 days from the beginning of experiment, the three groups of rats were sacrificed by
29 inhalation of ether. The testis were then extracted, dissected , carefully washed with normal

1 saline and then fixed in 10% formalin. The fixed materials were embedded in paraffin wax and
2 sections of 5-micrometer thickness were cut. Slides were stained with Haematoxylin and Eosin
3 [Bancroft and Gamble,2008] and Masson's trichrome stains [Leong 1996] For light microscopic
4 examination .

5 **Immunohistochemical studies:**

6 **Vaux et al (1988)** discovered the anti-apoptotic activity of Bcl-2 protein. We tried to detect this
7 protein by incubation of testicular tissues with antibodies directed against Bcl-2.

8 Avidin–Biotin–Peroxidase method was used for the Immunohistochemical analyses
9 [Jahnukainen et al., 2004]. Testicular tissues were deparaffinized , washed with phosphate
10 buffer solution (PBS) and incubated in 3% H₂O₂for 10 min,then incubated with 1% untreated
11 goat serum for 1 h. Testicular sections were washed in PBS. The monoclonal antibody was
12 applied overnight in humid medium at room temperature followed by the biotinylated
13 secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite
14 ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min
15 at room temperature as chromogenic and slides were then counterstained with hematoxylin,
16 dehydrated, and covered by coverslips. Bcl 2 was detected using an antihuman Bcl2 monoclonal
17 then Bcl-2 positive spermatogenic cells were evaluated under light microscope.

18 **Morphometric study:**

19 The mean area percentage of collagen fibers deposition and Bcl-2 immuno-expression were
20 quantified in five images from five non-overlapping fields of each rat using Image-Pro Plus
21 program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

22 **Statistical analysis :**

23 The data collected from the experiment was recorded and analyzed using IBM SPSS Statistics
24 software for Windows, Version 20 (IBM Corp., Armonk, NY, USA). One-way analysis of variance
25 (ANOVA) with Post Hoc LSD test was used to compare differences among the groups. In each
26 test, the data was expressed as the mean (M) value, standard deviation (SD) and differences
27 were considered to be significant at $P < 0.01$.

28 **Results:**

29 The testis of rats in the control group showed normal histological structure of the seminiferous
30 tubules . The tubules are lined by spermatogenic series which are arranged in layers with sertoli
31 cells in between. The lumens are filled with sperms. Interstitial tissue was filled with interstitial
32 cells(Leydig cells),(Figs. 1&2)

1 Exposure to Glyphosate lead to severe testicular degeneration and distortion . Seminiferous
2 epithelium showed degeneration of the spermatogonia , many seminiferous tubules showed
3 germ cell disorganization with necrotic cellular debris. Some other seminiferous tubules
4 appeared markedly necrotic, with degeneration of epithelial cells and only remnants of the
5 basement membrane. Other seminiferous tubules showed irregular shape and disarranged
6 epithelial cells. The interstitial tissue showed congestion and inflammatory cells(Figs. 3&4)

7 Testis of animals treated with allium cepa and glyphosate showed a more or less preserved
8 normal histological structure and a high number of germ cell layers .The histopathological
9 alterations were less prominent than those in the testis of rats treated with glyphosate only

10 (Figs.5&6)

11 Masson' s trichrome staining revealed normal testicular capsule and interstitial connective
12 tissues in the control group (Fig.7) .It showed less collagen fibers deposition in the testicular
13 capsule, in the basal lamina and in the interstitial tissues in group III (Fig.9) when compared to
14 group II (Fig.8).

15 The present study showed that glyphosate induced testicular apoptosis as indicated by a
16 decrease in Bcl-2 in germ cells (Fig.11). While treatment by allium cepa with glyphosate showed
17 a high reaction of Bcl 2 (Fig.12) nearly similar to that in the control group (Fig.10)

18 **Morphometric results:**

19 The mean area percentage of collagen fibers deposition for all groups was represented in
20 table (1) and histogram (1). There was a significant increase of collagen fibers deposition
21 ($P<0.01$) in group II compared with groups I and III. The mean area % of Bcl-2 immuno-
22 expression for all groups was represented in table (2) and histogram (2). There was a
23 significant increase in Bcl-2 immuno-expression ($P<0.01$) in groups I and III compared with
24 group II.

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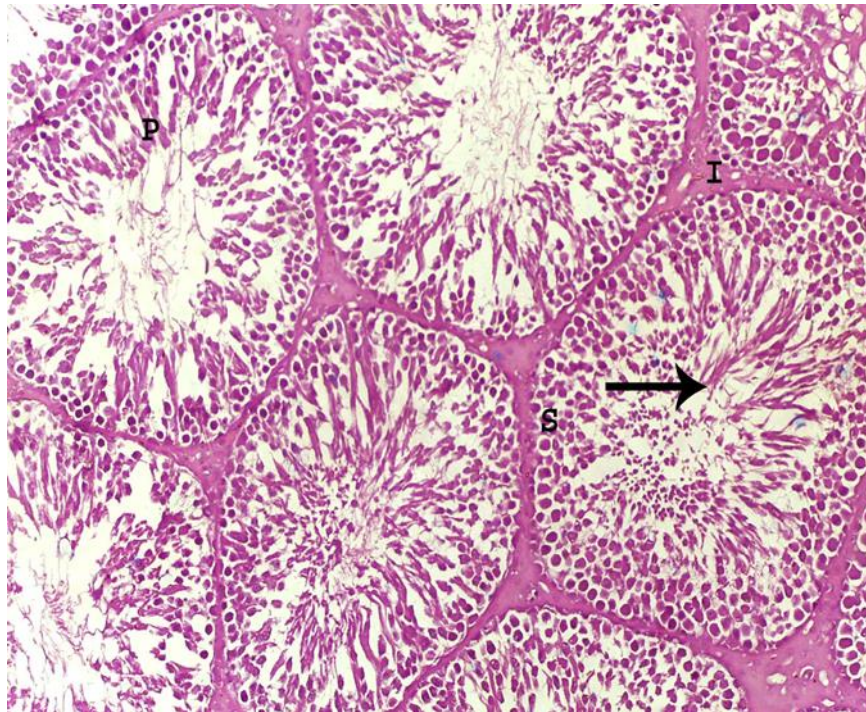
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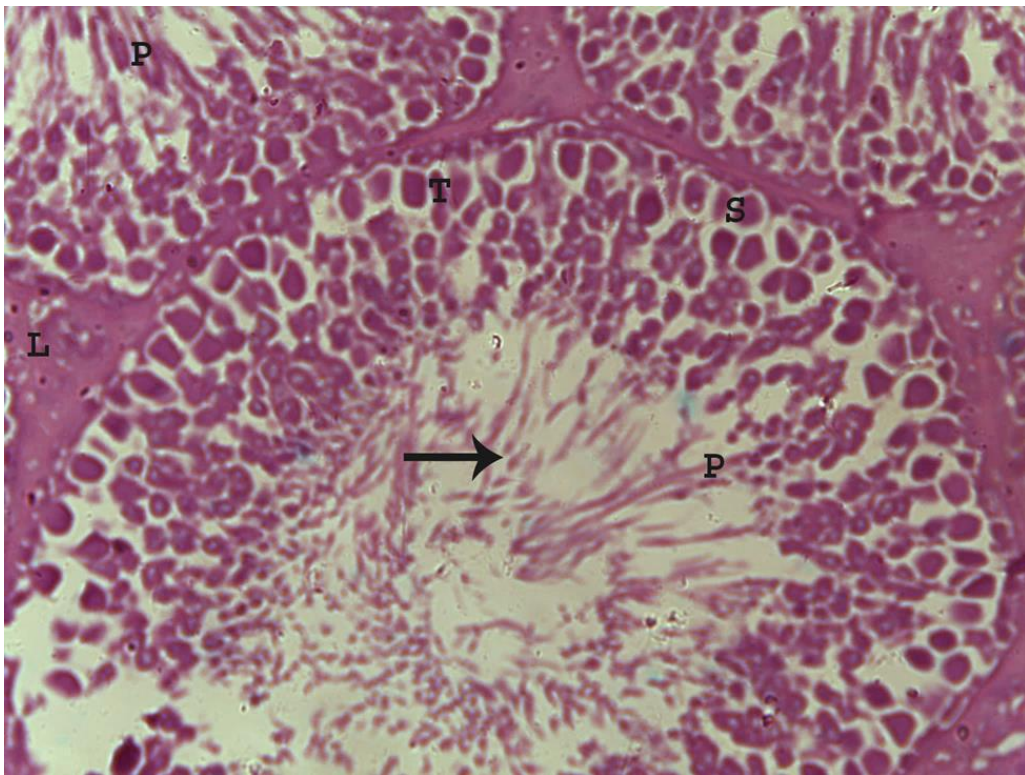
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9 Fig.(1)

10 photomicrograph of a section in an adult control rat testis; showing the
11 seminiferous tubules. The tubules contain the different stages of spermatogenic
12 cells (s), elongated sperms (P) are also seen in the lumen of the tubules, also note
13 the normal interstitial tissue showing Lydig cells(I) between the tubules. (Hx &E
14 200)

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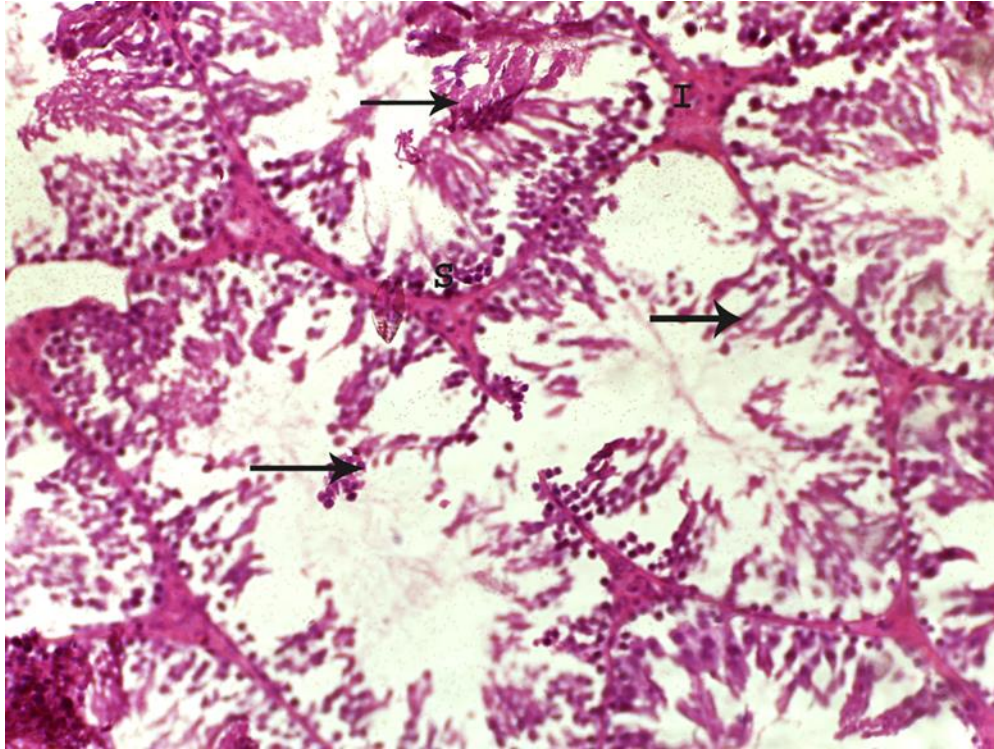
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8 **Fig.(2)**

9 **A photomicrograph of a section in an adult control rat testis showing**
10 **spermatogenic cells in layers (s) ,normal sertoli cells(T) and sperms (P) in the**
11 **normal seminiferous tubules.The interstitial space is normal showing Leydig cells**
12 **(L) .Notice the lumen is filled with sperms (arrow) (Hx&E x400)**

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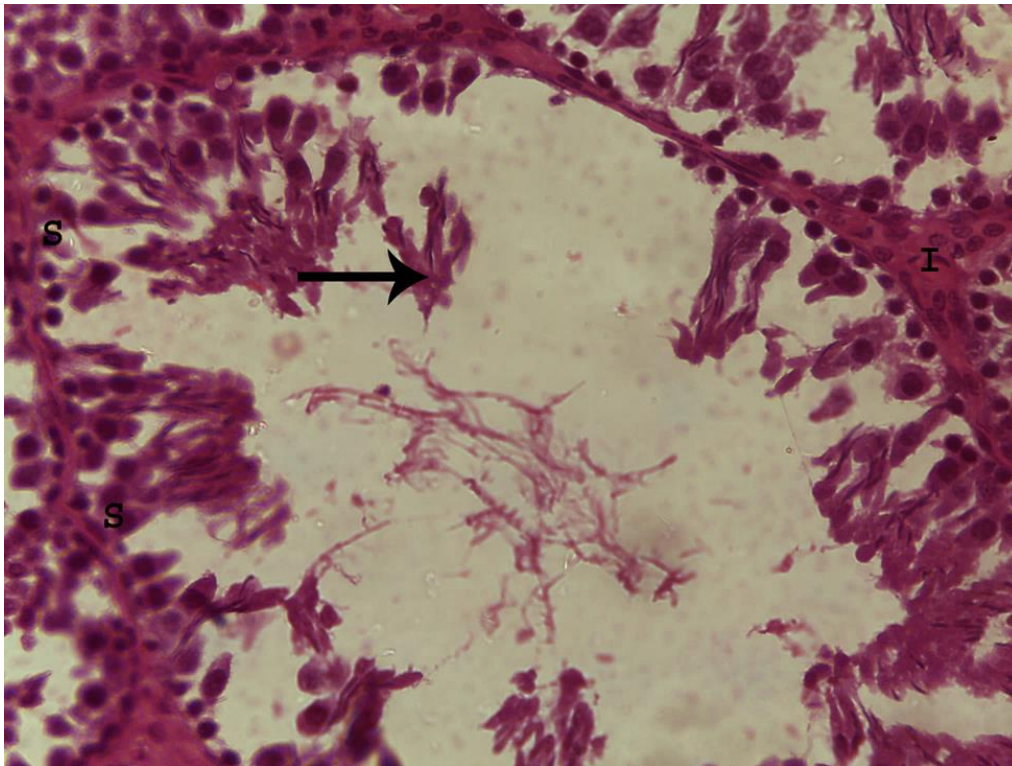
4 **Fig.(3)**

5 **A photomicrograph of a section in an adult rat testis treated with glyphosate**
6 **showing the seminiferous tubules with reduced number of spermatogenic cells.**
7 **Degenerative changes appeared in the spermatogenic cells (s) , and the lumens**
8 **dilated with few fragmented sperms in the lumen(arrow) . Interstitial tissues**
9 **showed inflammatory cells(I)**

10 **(Hx&E x200)**

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3 **Fig.(4)**

4 **A photomicrograph of a section in an adult rat testis treated with glyphosate**
5 **showing the seminiferous tubules with degeneration of spermatogenic**
6 **cells(S)and no sperms in the lumen . the lumen show degenerated cells (arrow)**
7 **The interstitial spaces showing inflamatory cells(I) and degeneration (Hx &E 400)**

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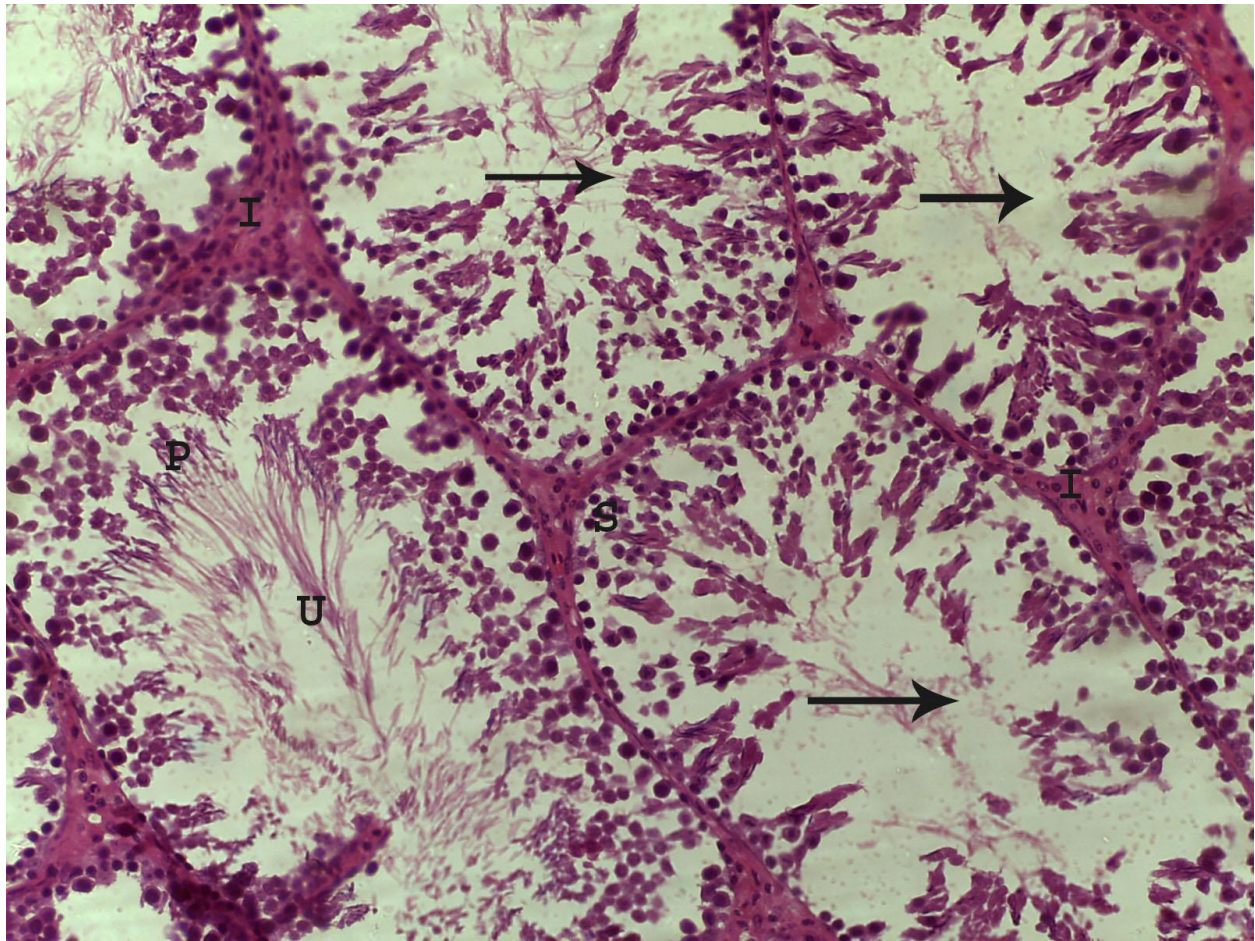
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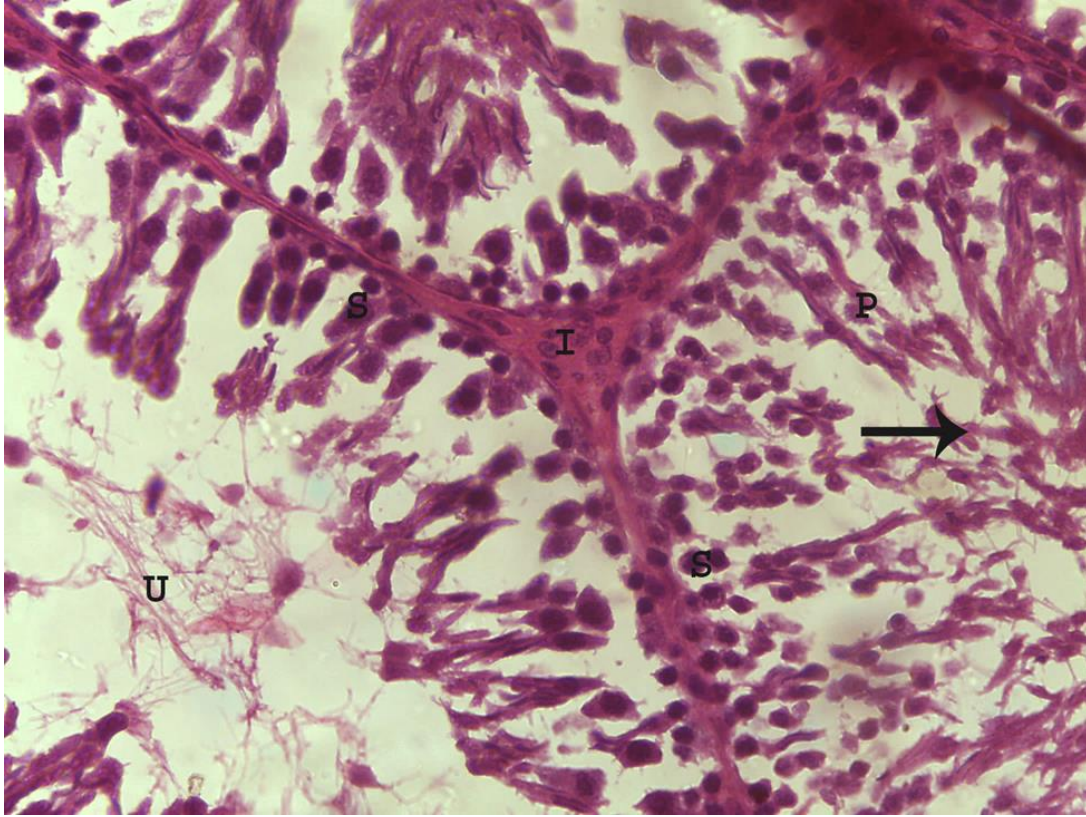
3 **Fig.(5): A photomicrograph of a section in an adult rat testis treated with**
4 **glyphosate and allium cepa showing slight regeneration of some seminiferous**
5 **tubules which showed regeneration of spermatogonia(S) . Some tubules were**
6 **regenerated (U) with normal apperance of sperms(p) others had degeneration**
7 **(arrow) .The interstial tissue showed inflamatory cells(I) (Hx&E x200)**

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3 **Fig.(6)**

4 **Aphotomicrograph of a section in an adult rat testis treated with glyphosate**
5 **and allium cepa showing slight regeneration of some seminiferous tubules**
6 **while others were regenerated (U) with normal spermatogonia(S) and normal**
7 **appearance of sperms(p) . Others were still degenerated (arrow) .The interstial**
8 **tissues showed inflammatory cells(I)**

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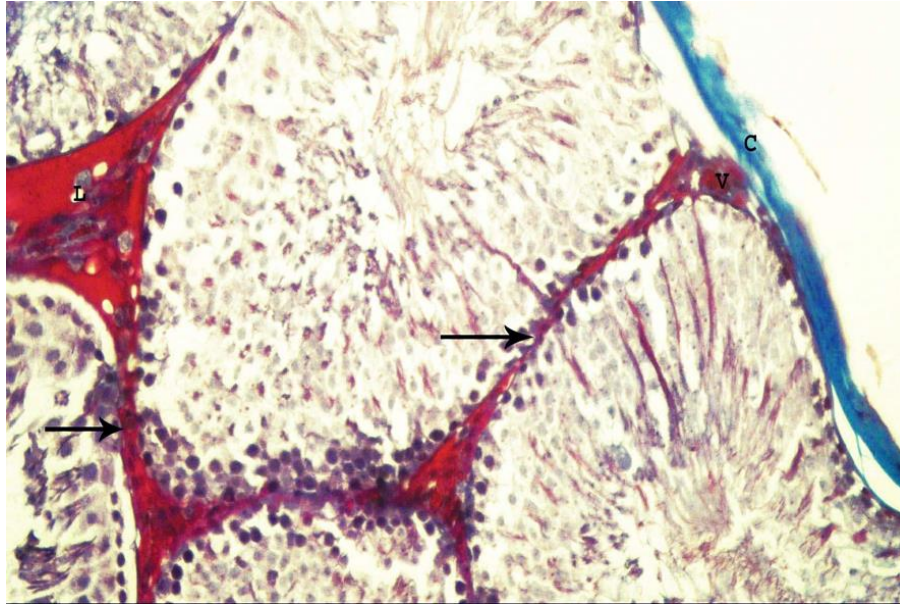
(Hx&E x400)

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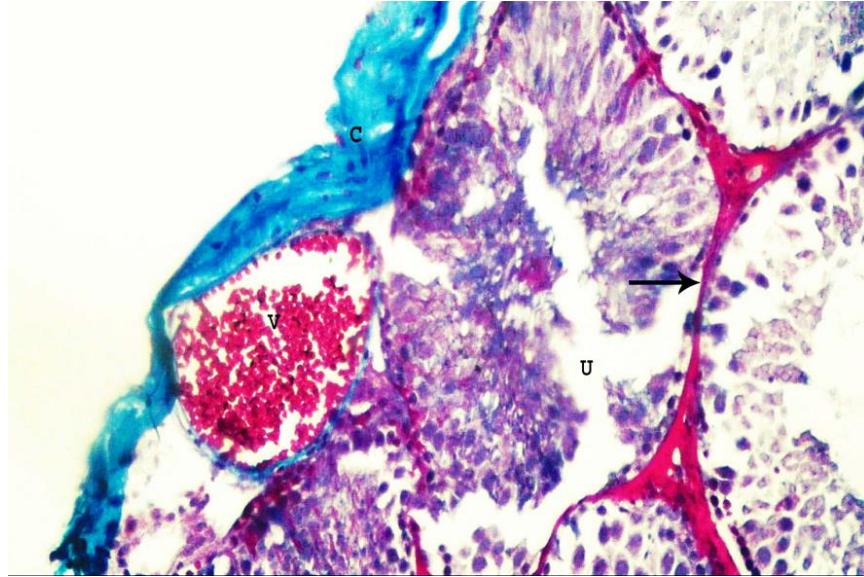
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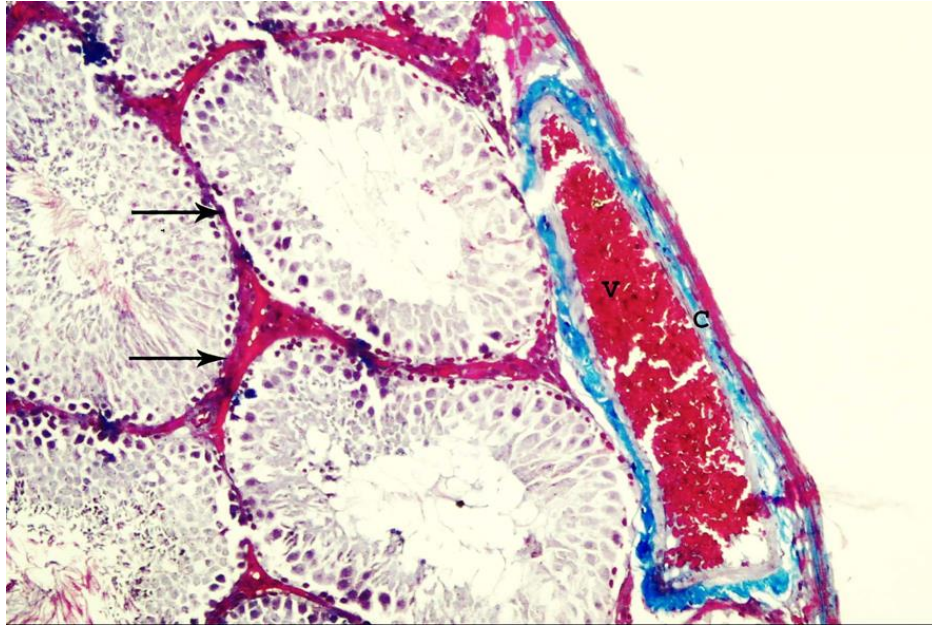
Fig. (7):
Aphotomicrograph of a section in an adult rat of Control group showing a normal distribution of collagen fibers in the testicular capsule(c), vessels of tunica vasculosa (v) , basal lamina(arrow) and interstitial tissues (i) .

(Masson' s Trichome x400).



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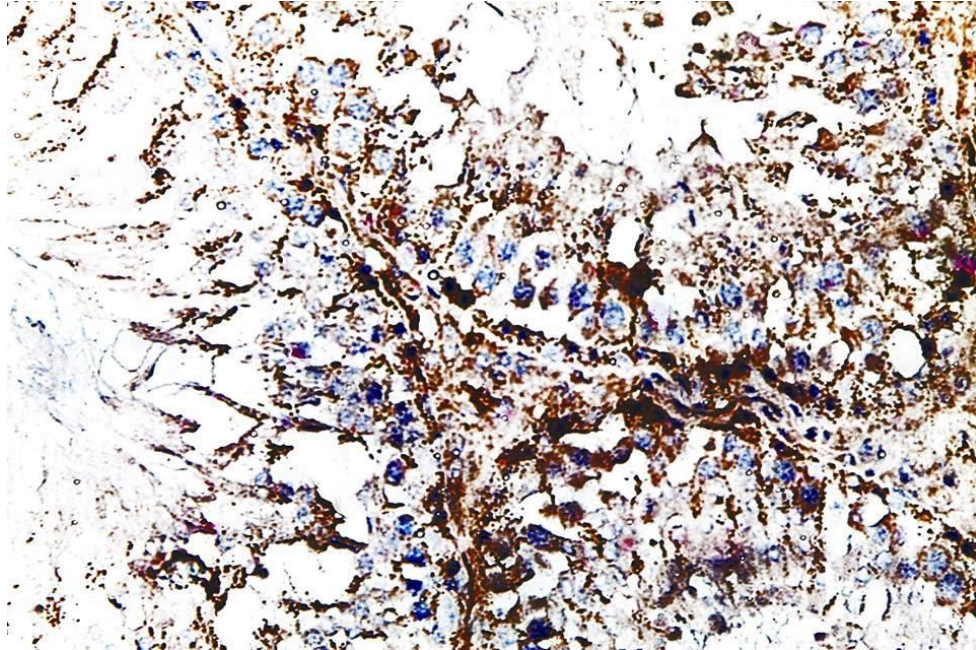
Fig. (8):
A photomicrograph of a section in an adult rat treated with glyphosate
Showing a marked increase of the collagen fibers deposition in the wavy
testicular capsule(c), around a blood vessel in the tunica vasculosa (V) ,the basal
lamina (arrow) and in the interstitial tissues (i).Notice the degenerated tubules
(U) (Masson' s Trichome x400).



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Fig. (9):
A photomicrograph of a section in an adult rat treated with glyphosate & Allium cepa showing less collagen fibers deposition in the testicular capsule (c), in the basal lamina (arrow), in the interstitial tissues (i) and around blood vessels in tunica vasculosa (v) .

(Masson' s Trichome x 400).



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3 **Fig. (10):**

4 **A photomicrograph of the seminiferous tubules of control rat showing a high**
5 **reaction of Bcl 2.**

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(Bcl 2 Immunostaining 400)

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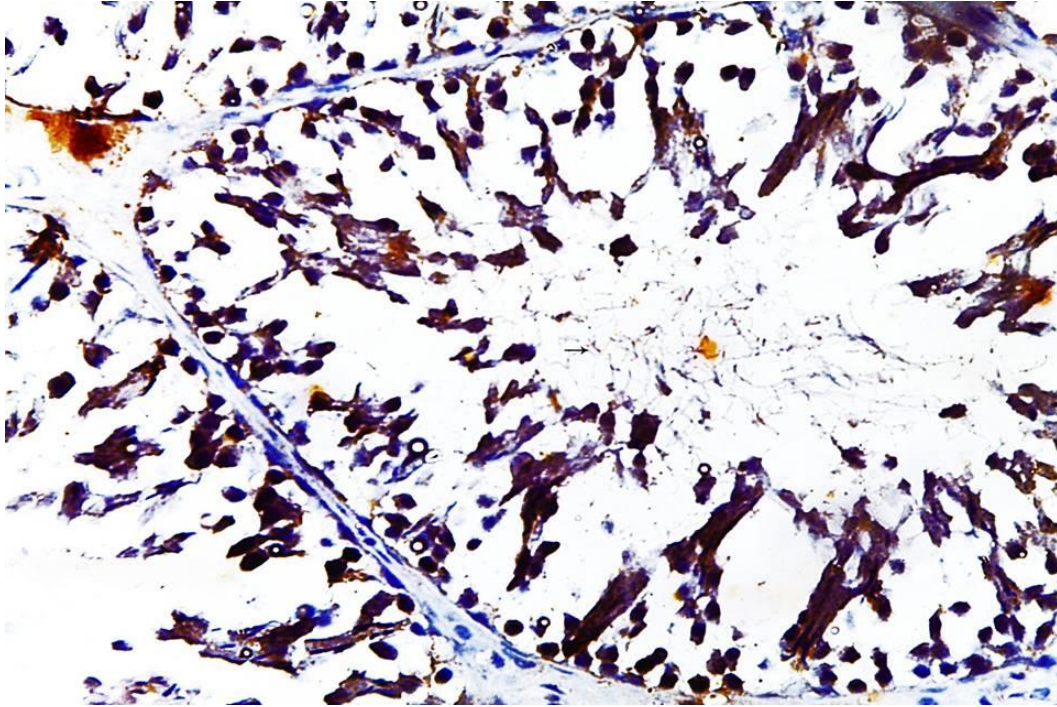
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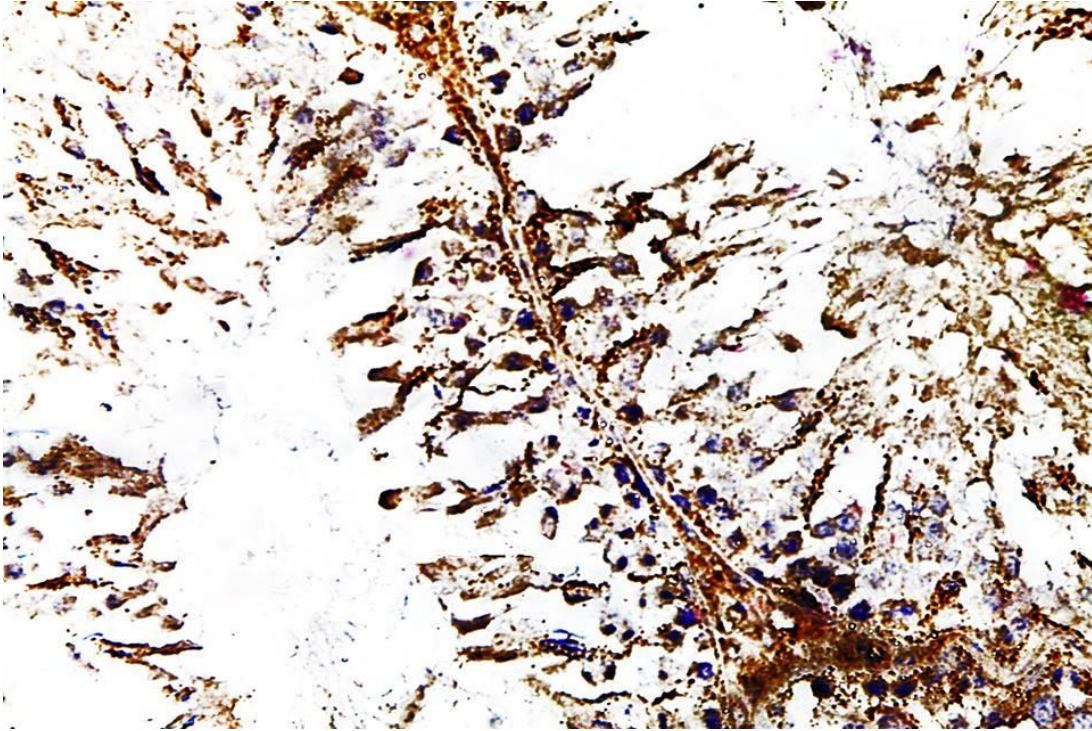
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5 **Fig . (11)**

6 **A photomicrograph of the seminiferous tubules of rat exposed to glyphosate**
7 **showing a low reaction of Bcl 2 .**

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(Bcl 2 Immunostaining 400)



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2 **Fig.(12)**

3 **A photomicrograph of the seminiferous tubules of rat treated by glyphosate**
4 **and allium cepa showing a high reaction of Bcl 2 .**

5 **(Bcl 2 Immunostaining 400)**

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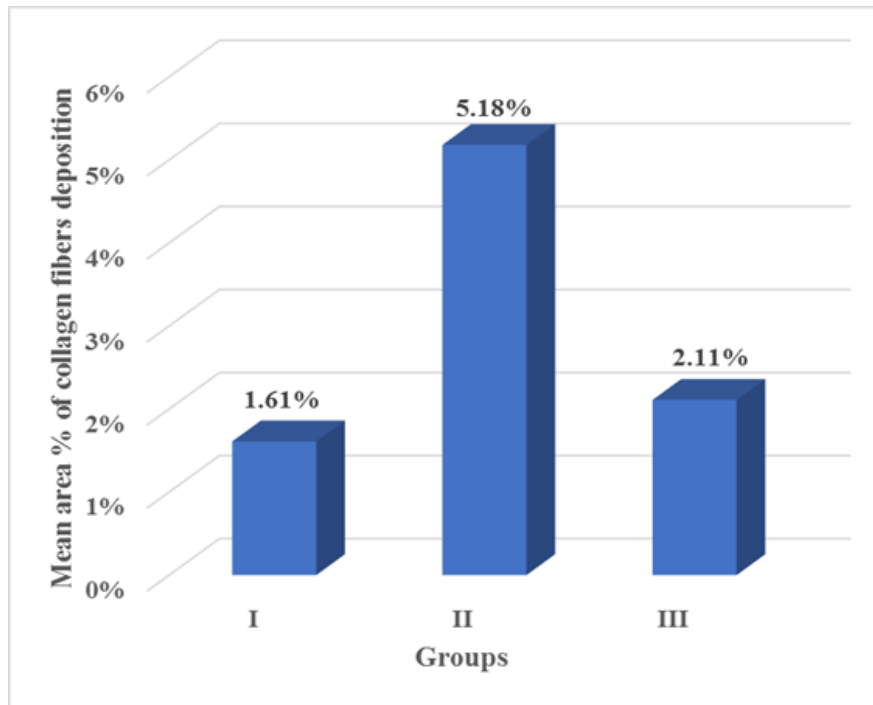
1 Table (1): Showing the mean area %, SD of collagen fibers deposition in groups I, II and III with a comparison
2 between all groups by Post Hoc LSD test.

	Group I	Group II	Group III
Mean area %	1.61%	5.18%	2.11%
SD	0.3385	0.3918	0.5006
Significance at P < 0.01	b	a,c	B

3 a=sig & group I b=sig & group II c=sig & group III

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7 Histogram (1): Showing the mean area % of collagen fibers deposition in groups I, II and III.

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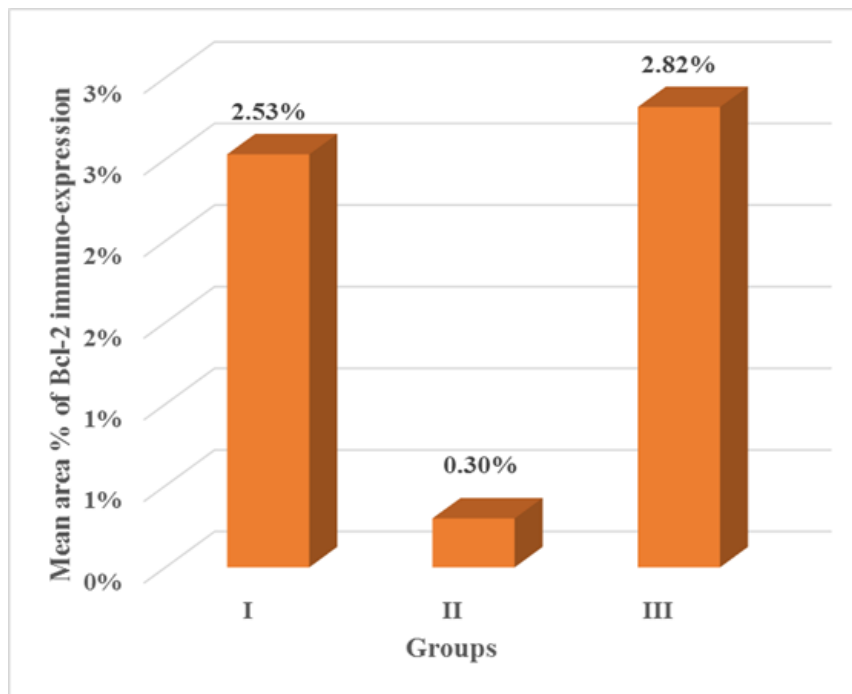
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1 **Table (2): Showing the mean area %, SD of Bcl-2 immuno-expression in groups I, II and III with comparison**
2 **between all groups by Post Hoc LSD test.**

	Group I	Group II	Group IV
Mean area %	2.53%	0.30%	2.82%
SD	0.3721	0.1027	0.2910
Significance at P < 0.01	b	a,c	b

3 a=sig & group I b=sig & group II c=sig & group III

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6 **Histogram (2): Showing the mean area % of Bcl-2 immuno-expression in groups**
7 **I, II and III.**

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1 **Discussion:**

2 In the present study, GP administrated at a dose of 125 mg /kg, body weight by oral gavage,
3 once a day for 30 days resulted In an irregularity in the shape of the seminiferous tubules (STs)
4 with a decrease in the number of primary spermatocytes and round spermatids in STs . **Shi &**
5 **Sharma (2011)** observed a loss of sperms, degeneration of interstitial cells and destruction of
6 all kind of GE series in rats exposed to OPC.

7 According to previous reports, the Glyphosate was able to inhibit the non-specific esterase
8 activity in Leydig cells, which inhibits steroidogenesis that in turn can result in inhibition of
9 testosterone synthesis (**Walsh et al., 2000**). It has been observed that glyphosate caused
10 testicular damage, including tubular necrosis and interstitial congestion, in rats,according to
11 **Ikpeme et al. (2012)**.

12 An earlier study carried out by **Ikpeme et al. (2010)** has established the adverse effect of
13 glyphosate administration on the hormones involved in spermatogenesis, hence its potential to
14 induce infertility in male mammals. Recently, it has been observed that glyphosate led to
15 oxidative stress and necrosis in rat testis, as a result of calcium overload, occurring through the
16 opening of L-type voltage- dependent calcium pump and calcium influx (**de Liz**

17 **Oliveira Cavalli et al.,2013; Samsel and Senef, 2013**). Our results showed that the Sertoli cells
18 were severely degenerated and the junction between germinal epithelium cells and Sertoli cells
19 was disrupted.

20 The cellular stress response and/or the depleted antioxidant defenses could contribute to the
21 Sertoli cell disruption; that could impact spermatogenesis and thus male fertility (**de Liz**
22 **Oliveira Cavalli et al., 2013**).

23 **Fattahi et al., (2009)** found that OPCs ,in addition to changing of hormonal levels, affect the
24 biochemical functions of the cells in the genital tract.

25 During spermatogenesis, apoptosis in testicular germ cells is recognized as an important
26 physiologic mechanism to limit the germ cell population to numbers that the Sertoli cells can
27 support (**Billig et al., 1995**). Regulation of germ cell apoptosis in the normal testis is controlled
28 by the Bcl- 2 family (**Woolveridge and Morris, 2000**).

29 Immunohistochemical observations in the present study revealed that there was a significant
30 increase in Bcl-2 immuno-expression ($P < 0.01$) in groups I and III compared to group II. These
31 were in accordance with the results of **Yu et al., (2009)** who reported that the expression of Bcl-
32 2 significantly decreased when the apoptosis rate significantly raised. The results also goes with

1 those of **Sakr, and Al-Amoudi (2012)** who reported that the stress sources as irradiation, toxins
2 and oxidative stress can affect the members of Bcl-2 family in the cell.

3 The present results clearly indicate that administration of *Allium cepa* (onion) in a dose of 1ml /
4 100 gm with exposure to glyphosate has a good effect on spermatogenesis in rats. These effects
5 could be related to vitamins(vitamin C) and flavonoids of onion such as quercetin which is a
6 natural antioxidant (**McAnlis et al, 1999**). Flavonoid quercetin and daizein have protective
7 effects on cadmium or polychlorinated biphenyls-induced oxidative damage in mice testes (**Bu**
8 **et al, 2006**).

9 Studies showed that C, E, and B vitamins are useful in reducing the poisonous effects on tissue
10 of the testes (**Yang et al, 2006**)

11 Previous studies found that *Allium cepa* also protects DNA and other important molecules from
12 oxidation and damages, and could improve sperm health parameters, increasing the rate of
13 fertility in men (**Rajeev and Narmada, 2006 & Yang et al. 2006**). The antioxidant effect of *A.*
14 *cepa* has been associated with reduced lipid peroxidation index malondialdehyde (MDA) and
15 increased superoxide dismutase (SOD), (**Ige et al, 2011, Guercio et al., 2014**).

16 **Conclusion:** The *Allium cepa* extract has a protective effect on testes of rat

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10 **Legends of figures:**

- 11 Fig.(1) :A photomicrograph of a section in an adult control rat testis.(Hx&Es200)
 12 Fig.(2): A photomicrograph of a section in an adult control rat testis. (Hx&Es 400)
 13 Fig.(3): A photomicrograph of a section in an adult rat testis treated with glyphosate
 14 (Hx&Es200)
 15 Fig.(4) :A photomicrograph of a section in an adult rat testis treated with glyphosate
 16 (Hx& Es 400)
 17 Fig.(5): A photomicrograph of a section in an adult rat testis treated with glyphosate and allium
 18 cepa (Hx&Es 200)
 19 Fig.(6) :A photomicrograph of a section in an adult rat testis treated with glyphosate and allium
 20 cepa (Hx&Es 400)
 21 Fig. (7):A photomicrograph of a section in an adult rat of Control group . (Masson's Trichome
 22 x400)
 23 Fig. (8):A photomicrograph of a section in an adult rat treated with glyphosate.(Masson's
 24 Trichome x400)
 25 Fig. (9):A photomicrograph of a section in an adult rat treated with glyphosate &Allium cepa
 26 (Masson,s Trichome x400)
 27 Fig. (10): A photomicrograph of the seminiferous tubules of control rat.(Bcl 2 Immunostaining
 28 400)
 29 Fig . (11) :A photomicrograph of the seminiferous tubules of rat exposed to glyphosate . (Bcl 2
 30 Immunostaining 400)
 31 Fig. (12) :A photomicrograph of the seminiferous tubules of rat treated by glyphosate and
 32 allium cepa .(Bcl 2 Immunostaining 400)

التاثيرات الواقيه لاليوم سييا على الخصيه من الفئران المعرضة للجليفوسات

نجلاء على صابر سرج, ساميه محمود مناوى ,كمال مصطفى كمال

قسم التشرييح والاجنة كلية الطب جامعة بنها

المخلص

مقدمة: يعتبر الجليفوسات(فوسفونوميثيل جلايسين) من مبيدات الاعشاب العضويه الفوسفاتيه الاكثر استخداما على نطاق واسع. كما يعتبر اليوم سييا المعروف باسم البصل من النباتات التي ثبتت من الدراسات السابقة ان له خصائص مضادة للاكسده فى كل من الفئران و البشر.

الهدف من الدراسة: يهدف هذا البحث إلى دراسه التأثير النسيجى والمناعى للجليفوسات كاحد المركبات العضويه الفوسفاتيه على الخصيه فى ذكور الفئران وتعيين التأثير الوقائى لاليوم سييا على الخصيه فى الفار الذى تعرض للجليفوسات

المواد والطرق: و قد اجرى هذا البحث على 30 فأرا من ذكور الفئران البيضاء البالغين و قد تم تقسيمهم إلى ثلاث (مجموعات: المجموعة الأولى (10 فئران): مجموعة ضابطه اعطيت ماء مقطر (0.2 مل /يوم

.. المجموعة الثانية (10 فئران): اعطيت جليفوسات بجرعة (125ملجم /كجم) من وزن الجسم

المجموعة الثالثة (10 فئران): اعطى كل منهم (1مل/100جرام) من وزن الجسم قبل ساعتين من اعطاء جليفوسات بجرعة 125 ملجم /كجم) من وزن الجسم . وتم اعطاءجميع الادويه وكذلك الماء المقطر يوميا من خلال انبوبة بالفم لمدة30يوم

.وقد تم إستئصال الخصى وأخذت منها عينات تم دراستها هستولوجيا ومناعيا بواسطة الميكروسكوب الضوئى

النتائج:اظهرت تلك الدراسه بفحص أنسجة الخصيه بالميكروسكوب الضوئى ان الجليفوسات فى المجموعه الثانيه تسبب فى انحطاط جميع طبقات الخلايا الجرثوميه مع وجود إحتقان فى الوريد وزيادة الياف الكولاجين فى الكبسول المحيط بالخصية، كما اوضحت النتائج المناعيه انخفاض البروتين المضاد لموت الخلايا المبرمج

كما وجد ان اعطاء اليوم سييا فى المجموعه الثالثه ادى الى تحسن جزئيا للتاثير الضار للجليفوسات على الانابيب المنوية

الاستنتاج: وبهذا نستطيع أن نستنتج من هذا البحث ان اليوم سييا يحمى الخصيه من التاثير الضار للجليفوسات