Original Article	**Protective Effects of Allium Cepa on Testis of Rats Exposed to GlyPhosate**

Naglaa A .S. Sarg, Samia M. Manawy and Kamal M. Kamal
Department of Anatomy and Embryology, Faculty of Medicine, Benha University, Egypt

ABSTRACT

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

Aim of Study: Is to investigate the histological, and immunohistochemical effects of Glyphosate (GP) as an Organophosphrous compound (OPC) on rat testis and to assess the protective effect of Allium cepa on testis of rats exposed to glyphosate.

Materials and Methods: This study included 30 adult male Albino rats divided into 3groups. Group I (Control group): Each rat received distilled water(0.2 ml/day). Group II: Each rat received glyphosate at a dose of 125 mg /kg, body weight. Group III: Each rat was given Allium cepa (AcE) 1 ml/100 g BW two hours before administration of GP at a dose of 125 mg /kg body weight. All the drugs as well as distilled water were given daily by oral gavages for 30days.

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

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

Received: 24 June 2017, **Accepted:** 15 July 2017

Key Words: Allium cepa, antiapoptotic protein(Bcl2), glyphosate, immunohistochemistry.

Corresponding Author: Nagla Ali Saber Sarg, Department of Anatomy and Embryology, Faculty of Medicine, Benha University, Egypt, **Tel.:** +20 12250 51717, **E-mail:** naglasarg@hotmail.com

The Egyptian Journal of Anatomy, ISSN: 0013-2446, Vol. 42 No. 1

INTRODUCTION

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

Although glyphosate is considered a low-toxic herbicide, recent studies have revealed toxic effects resulting from even low-dose commercial formulations ***(Benachour et al. 2007)***. The herbicide glyphosate is considered as a potential endocrine chemical disruptor which interferes with the production, release, transport, metabolism, binding, action and elimination of natural hormones responsible for the regulation of developmental processes ***(Kavlock et al., 1996)*** Organophosphrous compounds partially exert their pathological impacts via promotion of oxidative stress in reproductive tissue ***(Milatovic et al.,2006)***. Accordingly, OP agents increase oxidants by disrupting enzymatic and/or non-enzymatic antioxidant defenses as well as enhancing high energy consumption coupled with inhibition of oxidative phosphorylation ***(Razi et al.,2012)***. In addition, oxidative stress may cause degenerative alterations in sperm cells due to the high levels of polyunsaturated fatty acids (PUFA) in their plasma membrane ***(Agarwal and Allamaneni 2006)***. Imbalanced generation of oxidants affects the integrity of the sperm's DNA by causing elevated frequencies of single

 DOI:10.21608/EJANA.2022.249441

strand DNA (SS-DNA) and double-strand DNA (DS-DNA) breaks ***(Fraga et al.,1996)***.

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

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

MATERIALS AND METHODS

A- Animals

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

B- Drugs

1- Preparation of Allium cepa Extract

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

2- Glyphosate

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

Experimental design

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

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

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

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

Histopathological analyses

After 30 days from the beginning of experiment, the three groups of rats were sacrificed by inhalation of ether. The testis were then extracted, dissected, carefully washed with normal saline and then fixed in 10% formalin. The fixed materials were embedded in paraffin wax and sections of 5-micrometer thickness were cut. Slides were stained with Haematoxylin and Eosin ***(Bancroft and Gamble,2008)*** and Masson's trichrome stains ***(Leong 1996)*** For light microscopic examination .

Immunohistochemical studies

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

Avidin–Biotin–Peroxidase method was used for the Immunohistochemical analyses ***(Jahnukainen et al., 2004)***. Testicular tissues were deparaffinized, washed with phosphate buffer solution (PBS) and incubated in 3% H_2O_2 for 10 min,then incubated with 1% untreated goat serum for 1 h. Testicular sections were washed in PBS. The monoclonal antibody was applied overnight in humid medium at room temperature followed by the biotinylated secondary antibody for 15 min at 37°C and the ABC complex for 15 min at 37°C (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA). Diaminobenzidine (DAB) was applied for 20 min at room temperature as chromogenic and slides were then counterstained with hematoxylin, dehydrated, and covered by coverslips. Bcl 2 was detected using an antihuman Bcl2 monoclonal then Bcl-2 positive spermatogenic cells were evaluated under light microscope.

Morphometric study

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

Statistical analysis

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

RESULTS

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

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

Testis of animals treated with allium cepa and glyphosate showed a more or less preserved normal histological structure and a high number of germ cell layers .The histopathological alterations were less prominent than those in the testis of rats treated with glyphosate only (Figures 5,6)

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

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

Morphometric results

The mean area percentage of collagen fibers deposition for all groups was represented in (Table 1, Histogram 1). There was a significant increase of collagen fibers deposition ($P<0.01$) in group II compared with groups I and III. The mean area % of Bcl-2 immuno-expression for all groups was represented in (Table 2, Histogram 2). There was a significant increase in Bcl-2 immuno-expression ($P<0.01$) in groups I and III compared with group II.

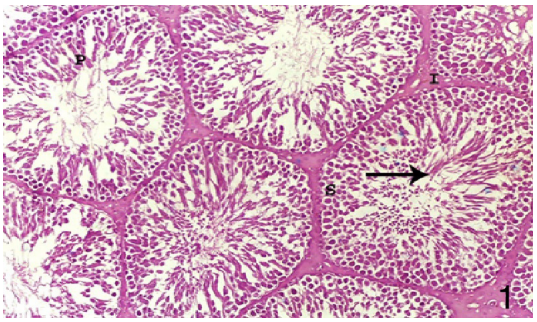


Fig. 1: photomicrograph of a section in an adult control rat testis; showing the seminiferous tubules. The tubules contain the different stages of spermatogenic cells (s), elongated sperms (P) are also seen in the lumen of the tubules, also note the normal interstitial tissue showing Lyedig cells(I) between the tubules. (Hx &E 200)

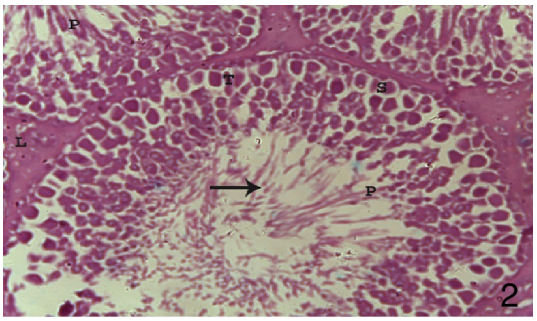


Fig. 2: A photomicrograph of a section in an adult control rat testis showing spermatogenic cells in layers (s) ,normal Sertoli cells(T) and sperms (P) in the normal seminiferous tubules. The intestinal space is normal showing lyding cells (L) .Notice the lumen is filled with sperms (arrow) (Hx&E x400)

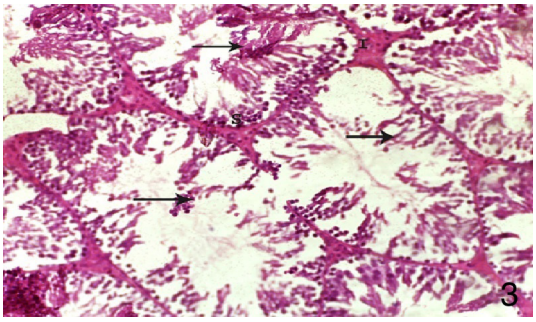


Fig. 3: A photomicrograph of a section in an adult rat testis treated with glyphosate showing the seminiferous tubules with reduced number of spermatogenic cells. Degenerative changes appeared in the spermatogenic cells (s) , and the lumens dilated with few fragmented sperms in the lumen(arrow) . Interstitial tissues showed inflammatory cells(I) (Hx&E x200)

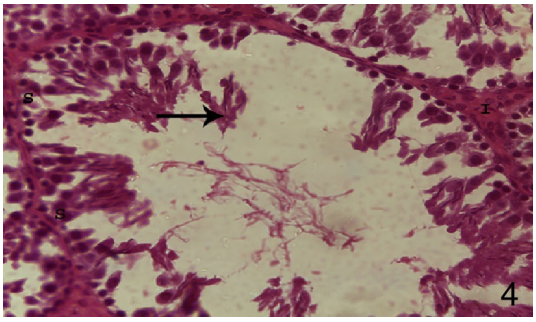


Fig. 4: A photomicrograph of a section in an adult rat testis treated with glyphosate showing the seminiferous tubules with degeneration of spermatogenic cells(S)and no sperms in the lumen . the lumen show degenerated cells (arrow) The interstitial spaces showing inflammatory cells(I) and degeneration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

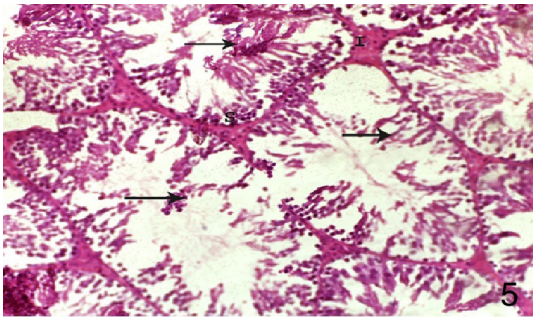


Fig. 5: A photomicrograph of a section in an adult rat testis treated with glyphosate and allium cepa showing slight regeneration of some seminiferous tubules which showed regeneration of spermatogonia(S) . Some tubules were regenerated (U) with normal appearance of sperms(p) others had degeneration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

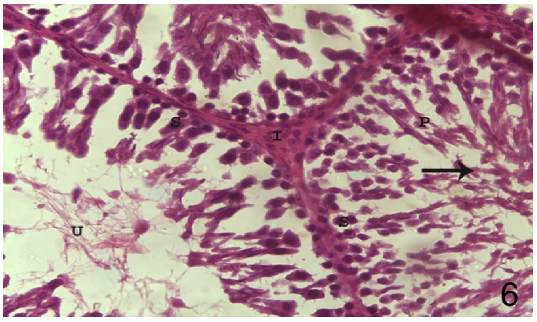


Fig. 6: Photomicrograph of a section in an adult rat testis treated with glyphosate and allium cepa showing slight regeneration of some seminiferous tubules while others were regenerated (U) with normal spermatogonia(S) and normal appearance of sperms(p). Others were still degenerated (arrow) .The interstitial tissues showed inflammatory cells(I) (Hx&E x400)

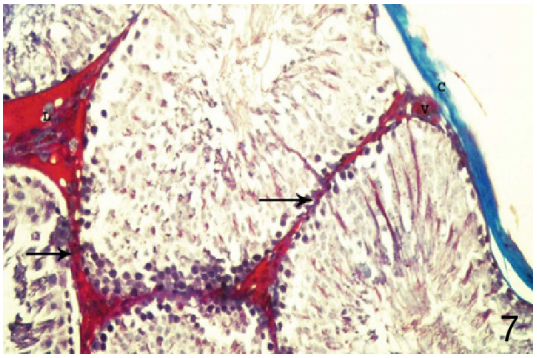


Fig. 7: Photomicrograph of a section in an adult rat of Control group showing a normal distribution of collagen fibers in the testicular capsule(c), vessels of tunica vasculosa (V) , basal lamina(arrow) and interstitial tissues (i). (Masson x400).

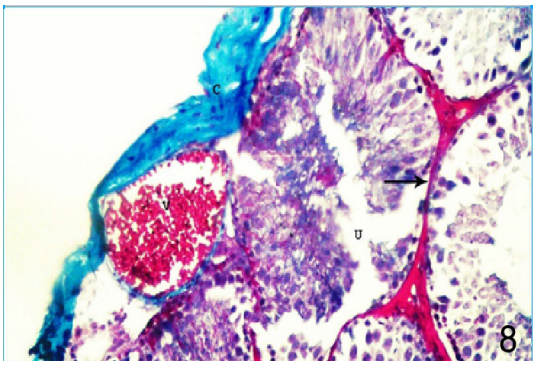


Fig. 8: Photomicrograph of a section in an adult rat treated with glyphosate Showing a marked increase of the collagen fibers deposition in the wavy testicular capsule(c), around a blood vessel in the tunica vasculosa (V), the basal lamina (arrow) and in the interstitial tissues (i). Notice the degenerated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

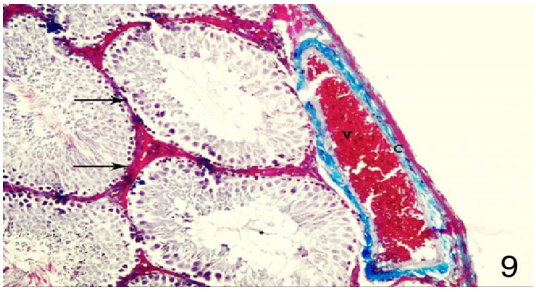


Fig. 9: A photomicrograph of a section in an adult rat treated with glyphosate & Allium cepa showing less collagen fibers deposition in the testicular capsule (c), in the basal lamina (arrow), in the interstitial tissues (i) and around blood vessels in tunica vasculosa (v). (Masson x 400).

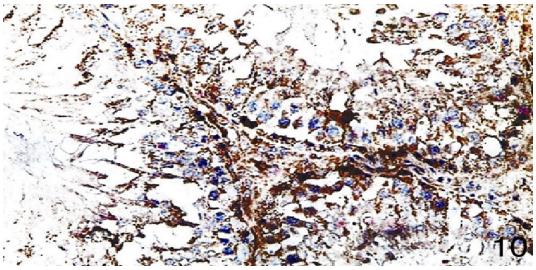


Fig. 10: A photomicrograph of the seminiferous tubules of control rat showing a high reaction of Bcl 2. (Bcl 2 Immunostaining 400)

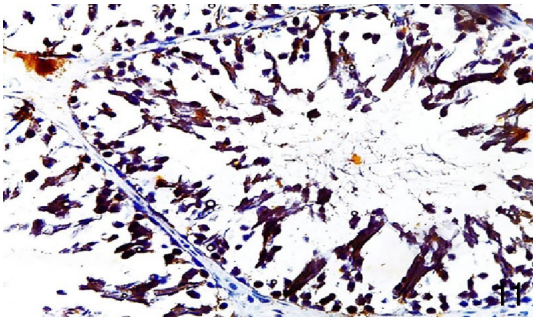


Fig. 11: A photomicrograph of the seminiferous tubules of rat exposed to glyphosate showing a low reaction of Bcl 2. (Bcl 2 Immunostaining 400)

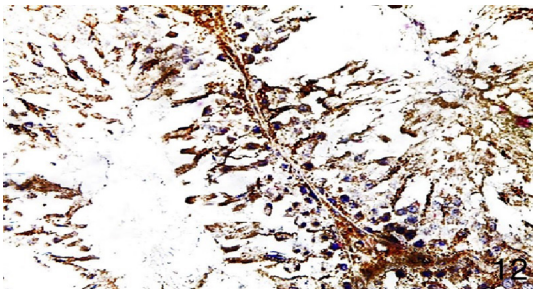


Fig. 12: A photomicrograph of the seminiferous tubules of rat treated by glyphosate and allium cepa showing a high reaction of Bcl 2. (Bcl 2 Immunostaining 400)

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

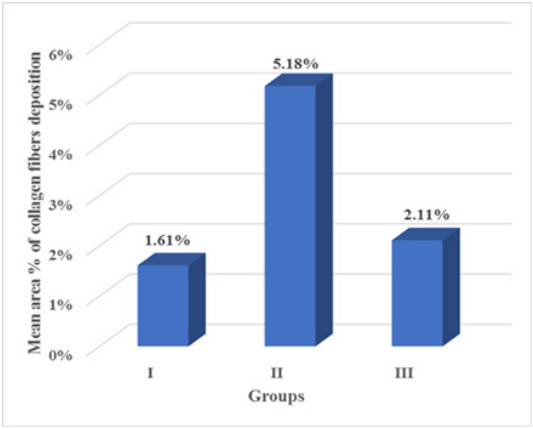
	Group I	Group I I	Group I I I
Mean area %	1.61%	5.18%	2.11%
SD	0.3385	0.3918	0.5006
Significance at $P < 0.01$	b	a,c	B

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

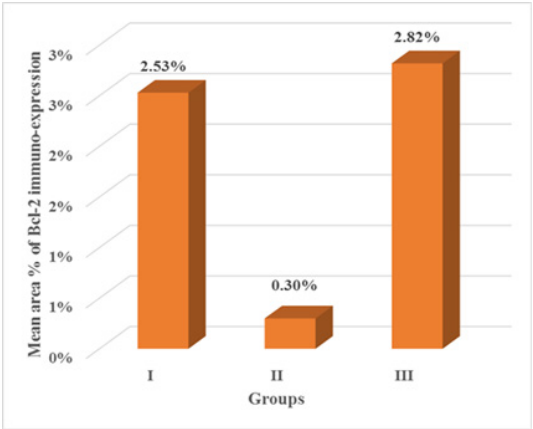
Table 2: Showing the mean area %, SD of Bcl-2 immuno-expression in groups I, II and III with comparison between all groups by Post Hoc LSD test

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

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



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



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

DISSCUSION

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

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

An earlier study carried out by Ikpeme *et al*. (2010) has established the adverse effect of glyphosate administration on the hormones involved in spermatogenesis, hence its potential to induce infertility in male mammals. Recently, it has been observed that glyphosate led to oxidative stress and necrosis in rat testis, as a result of calcium overload, occurring through the opening of L-type voltage- dependent calcium pump and calcium influx ***(de Liz Oliveira Cavalli et al.,2013; Samsel and Senef, 2013)***. Our results showed that the Sertoli cells were severely degenerated and the junction between germinal epithelium cells and Sertoli cells was disrupted.

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

Fattahi etal., (2009) found that OPCs ,in addition to changing of hormonal levels, affect the biochemical functions of the cells in the genital tract.

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

Immunohistochemical observations in the present study revealed that there was a significant increase in Bcl-2 immuno-expression (P<0.01) in groups I and III compared to group II. These were in accordance with the results of Yu *et al*., (2009) who reported that the expression of Bcl-2 significantly decreased when the apoptosis rate significantly raised. The results also goes with those of Sakr, and Al-Amoudi (2012) who reported that the stress sources as irradiation, toxins and oxidative stress can affect the members of Bcl-2 family in the cell.

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

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

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

CONCLUSION

The Allium cepa extract has a protective effect on testes of rat

CONFLICT OF INTERESTS

There are no conflicts of interest.

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التاثيرات الواقيه لاليوم سيبا على الخصيه من الفئران المعرضة للجليفوسات

نجلاء على صابر سرج، ساميه محمود مناوى، كمال مصطفى كمال
قسم التشريح والاجنة - كلية الطب - جامعة بنها

ملخص البحث

مقدمة: يعتبرالجليفوسات(فوسفونوميثيل جلايسين) من مبيدات الاعشاب العضويه الفوسفاتيه الاكثراستخداما على نطاق واسع. كما يعتبر اليوم سيبا المعروف باسم البصل من النباتات التي ثبت من الدراسات السابقه ان له خصائص مضادة للاكسده فى كل من الفئران و البشر.
الهدف من الدراسة: يهدف هدا البحث إلى دراسه التأثير النسيجى والمناعى للجليفوسات كاحد المركبات العضويه الفوسفاتيه على الخصيه فى ذكور الفئران وتعيين التاثير الوقائى للاليوم سيبا على الخصيه فى الفار الذى تعرض للجليفوسات.
المواد والطرق: وقد اجرى هدا 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 ا(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 لمدة 30 يوم وقد تم إستئصال الخصى وأخدت 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 سيبا فى المجموعة الثالثه ادى الى تحسن جزئيا للتااثير الضار للجليفوسات على الانابيب المنوية.
الاستنتاج: وبهذا نستطيع أن نستنتج من هذا البحث ان اليوم سيبا يحمى الخصيه من التاثير الضار للجليفوسا ت.