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Evaluation of the Effect of Edta Contaminated Food on the Fertility of Male Mice

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ABSTRACT

The present study was investigated the effects of Ethylenediaminet etraacetic acid(EDTA) contaminated food manipulation on the fertility of male mice the investigtion based on evaluation of physical semencharacteristics, andmorphometrical andhistopathological studies. ofForty healthy adult male Swiss Albino mice have been divided into four groups. The first group (the control) received only standard diet while the second to the fourth groups were supplemented daily with contaminated food with different EDTA concentrations, 0.5, 1.5 and 5 g/kg of food. The samples were collected after two experimental periods (four and eight weeks). Results showed that the EDTA manipulated groups exhibited significantly decrease of sperm parameters and testosterone concentration. Morphometric study of testis in different EDTA manipulated groups showed a significant decrease of primary spermatocytes and fertile seminiferous tubules in count as compared to control group. However, histological results showed decrement of spermatogeniclineage;declined spermatozoa formation and several necrotic areas. Also, some Sertoli cells and interstitial cells were degenerated. In addition, several blood vessels revealed dilation and congestion in between the interstitial cells. All the adverse alteration in the present results was proportional with the dose of EDTA manipulate on of mice and with the experimental period expansion. In conclusion, the adverse effects of EDTA as a chelating agent induced obviousinhibition of spermatogenic lineage and degeneration of interstitial cells leading to decrease the fertility of male mice.

Keywords:mice EDTA, spermatogenesis, male infertility, semen.

INTRODUCTION

Despite the diversity of habitat and cultures around the world, an augment in male infertility has been observed in recent decades and apparently it constitutes an international phenomenon. The true incidence of male infertility is unknown due to the enormous variability in infertility extensiveness. Spermatogenesis is a highly organized process of cell proliferation and terminal differentiation that leads to the formation of mature spermatozoa. Several external factors are susceptible to impair spermatogenesis and more specifically spermatogonia stem cells, such as cancer treatment, chemotherapy or radiotherapy, with possible transient or permanent spermatogenesis arrest (Dohle, 2010; Bin-Meferij and El-kott, 2015).

Humans have always been exposed to certain persistent chemicals, some pesticides, heavy metals, organic solvents, and, tobacco smoking, which are reported to have reproductive toxic potential for both sexes depending upon the dose and duration of the exposure.Naturally occurring environmental chemicals induce infertility in domestic animal species and may alter human reproductive function.Exposure to synthetic chemicals produces reproductive problems in a variety of vertebrate species *via* endocrine mechanisms (Sedha*et al*., 2015). Concerning the adverse effects of various environmental contaminants on the male reproductive system has been growing nowadays.

EDTA is purely a chemical that not occur in the natural It EDTA has been used extensively in medicine as a chelating agent for the removal of toxic heavy metals. The disodium salt of EDTA is a common component in many eye drops and contact lens wetting and cleaning solutions. EDTA is also used in a number of personal care and hygiene products, such as shampoos, liquid soaps, creams, and lotions. EDTA has been approved by the Food and Drug Administration as a food additive generally recognized as safe(Shackleford, 2004).

EDTA is a widespread organic pollutant. In combination with sodium and calcium EDTA has been used as the main therapeutic agent in case of lead poisoning for decades (Crinnion, 2011). As sodium iron EDTA has been shown to increase iron bioavailability in human diets and has been proposed for use as a fortificant in certain grain-based products including breakfast cereals and cereal bars. The typical concentration of use of EDTA is less than 2% with the other salts as mentioned by Lanigan and Yamarik (2002) at even lower concentrations. The lowest dose reported to cause a toxic effect in animals was 750 mg/kg/day.EDTA potentiates the mutagenic activity of chemical and physical agents in human and mice. It is also cytotoxic and weakly genotoxic, but not carcinogenic. Oral exposures to EDTA produced adverse reproductive and developmental effects in animals (Khalil*et al*., 2008; Lanigan and Yamarik, 2002).

The main aim of the present study was to evaluate the probable effects of feeding EDTA contaminated food on testis function, progression of spermatogenesis and fertility in adult male mice.

MATERIALS AND METHODS

Animals and experimental protocol

Total of 40 healthy adult male Swiss Albino mice were used in this study, weighing approximately 25-30 g. They were obtained from Helwan breeding farm, Egypt. The animals were housed in stainless cages and kept at temperature (22-25°C) in a light controlled room with a 12 hours alternating light/dark cycle. The animals received standard chow diet and water *ad libitum*. All care and procedures adopted for the present investigation were in accordance with the approval of the Institutional Animal Ethics Committee of National Research Center.

Mice were divided into four main groups (10 mice/each) as follows: 1) the control group, which fed on standard diet only. 2) The second group, where the male mice were fed on standard diet mixed with (EDTA 0.5 g/kg of food). 3) The third group, where the adult male mice were fed on standard diet mixed with (EDTA 1.5 g/Kg of food). 4) The fourth group, where the adult male mice were orally feed on standard diet mixed with (EDTA 5.0 g/kg of food). Each group was subdivided into two subgroups, according to the time of animal sacrifice after four and eight weeks.

Animal sacrifice and sample collection

At the time of sacrifice, mice were anesthetized and blood samples were collected for physiological investigations. From each scarified mouse, left testis were fixed in 10% neutral formalaldehyde for histopathological study for a time period of one day, and the right testis were processed for semen analysis.

Body weight gain

Body weight gain for each experimental group was recorded every other day. The mean of the body weight gain of each group was calculated at the end of every week.

Semen Analysis

Semen analysis was done for each animal in the studied groups. These include sperm count/ (million) andsperm vitality. Each semen specimen was taken by cutting tail portion of epididymis liberating sperm in 1ml of phosphate buffere saline solution for at least 15sec.

Morphometric assessments of testis function

In testis sections stained with haematoxlineHand eosin E, the number of seminiferous tubules was counted. The average ratio of fertile seminiferous tubules of EDTA groups was calculated in relation to the control group. The fertile tubules exhibited the regular arrangement of spermatozoa, spermatids, spermatocytes and spermatogonia. The number of primary spermatocytes was counted in 40 round seminiferous tubules of each mouse.

Histological examination

Sections of 4 to 5 µin thick were prepared using microtome, mounted on glass slide and stainedwith H and E and Masson Trichrome stain.

Statistical analysis

Results were expressed as mean ± standard error (S.E.). One-way analysis of variance (ANOVA) test as first carried out to test thesignificant differences between the mean values of all groups. If differences between groups were established, the values of the treated groups were compared with those of the control group by a multiple comparison t-test. A value of ($p < 0.05$) was interpreted as statistically significant.

RESULTS

Morphometeric study

Change of body weightgain

The body weight gain of the mice received EDTA as (0.5 and 1.5 g /kg of food) showed no significant($P > 0.05$) increase in comparison with the control group after four and eight weeks of exposure. While, the mice manipulated EDTA (5.0 g/kg of food) revealed significant ($P < 0.05$) increase in comparison with control after four and eight weeks of duration (Fig1).

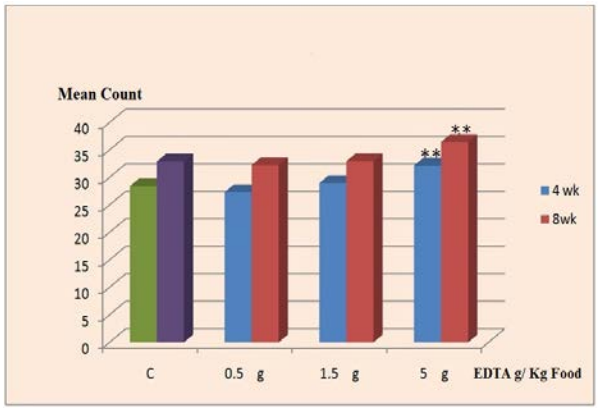

Fig. 1: The percentage of change in the body weight gain in control and different treated groups.= Highly Significance**

Morphometric Assessments of Testis Function

Primary spermatocytes count

Thepresent investigation showed highly significant ($P < 0.01$) decrement in the number of primary spermatocytes in EDTA manipulated groups received (0.5 and 1.5g/kg of food) after four weeks in comparison with control group. EDTA manipulated group received (5.0g/kg of food) showed significant ($P < 0.001$)decrease after four weeks comparing with control group. The EDTA manipulated group showed significant ($P < 0.05$) decrease in the lowest dose and sharp decrease ($P<0.001$) in the other two groups after eight weeks of the experimental exposure in comparisons with control (Fig.2).

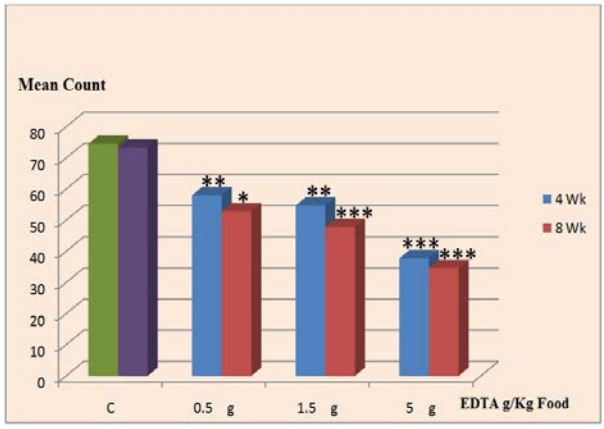

Fig. 2: percentage of change in primary spermatocytes count in control and different treated groups after 4 and 8 weeks.

*** = Significance.**= highly significant.***=very highly significant.**

Fertile seminiferous tubules count

EDTA manipulated group received (0.5 g/kg of food) showed non- significant change after four weeks when compared with control group, while the group received (1.5 and 5.0 g/kg of food) revealed very highly decrease ($P < 0.001$) in the fertile number of seminiferous tubules after four weeks comparing with

control group. While the number fertile seminiferous tubules showed significant (P< 0.05) decrement in EDTA group received (0.5g/kg of food) and very highly significant decrease in both groups received EDTA (1.5 and 5.0g /kg of food) comparing with control group (Fig.3).

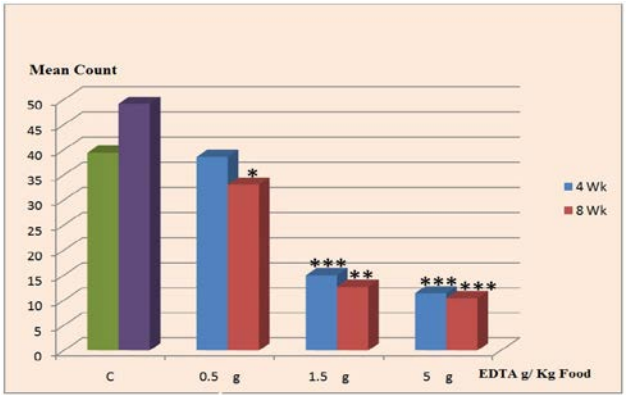

Fig. 3: Percentage of change in fertile seminiferous tubules count in control and different treated groups.

*** = Significance. ***= very highly significant**

Thickness of tunica albuginealayer (TAL)

The present study showed significant decrease in thicknessTALof EDTA manipulation group at dose (0.5g/kg of food), while the other groups (1.5 and 5.0g/kg of food) showed very highly significant (P< 0.001) decrease after eight weeks of exposure comparing with control (Fig4).

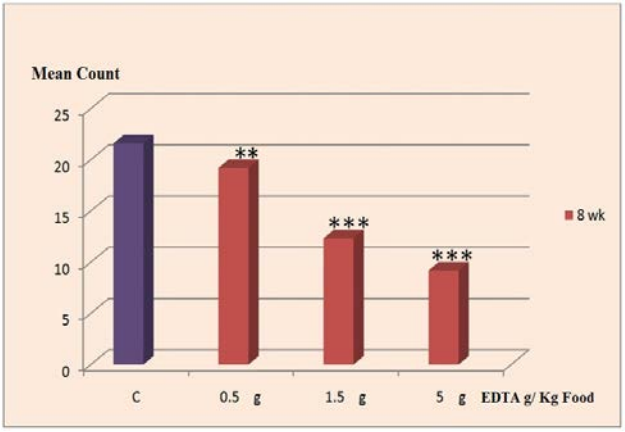

Fig 4: Tunica albuginea thickness layer stained with MT in control and different treated groups.

*** = Significance. ***= very highly significant**

Semen analysis

Sperm vitality

The present study showed significant decrease of EDTA manipulation group at dose (0.5g/kg of food), while the other groups (in sperm vitality 1.5 and 5.0g/kg of food) showed significant (P< 0.01) decrease after four weeks comparing with control. After eight weeks of EDTA manipulation the mice received EDTA (0.5 and 1.5g/kg of food) showed highly significant (P< 0.01) decrement while the mice received the highest dose of EDTA showed very highly significant (P< 0.001)decline when compared with control (Fig.5).

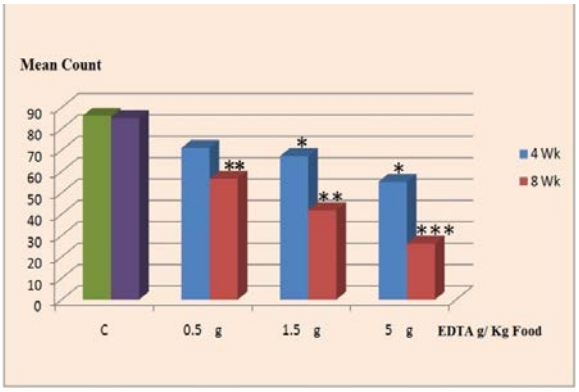

Fig. 5: Percentage of change of sperm vitality in control and different EDTA manipulated groups.

*** = Significance,**=highly significant,***=very highly significant**

Sperm count

The present study showed non- significant change in sperm count of EDTA manipulation group at dose (0.5g/kg of food), while the other groups (1.5 and 5.0g/kg of food) showed significant and highly significant decrease (P< 0.01, 0.001) after four weeks comparing with control. After eight weeks of EDTA manipulation the mice received EDTA (0.5 and 1.5g/kg of food) showed significant (P< 0.05) decrement while the mice received the highest dose of EDTA showed highly significant (P< 0.001) decline when compared with control **(**Fig.6).

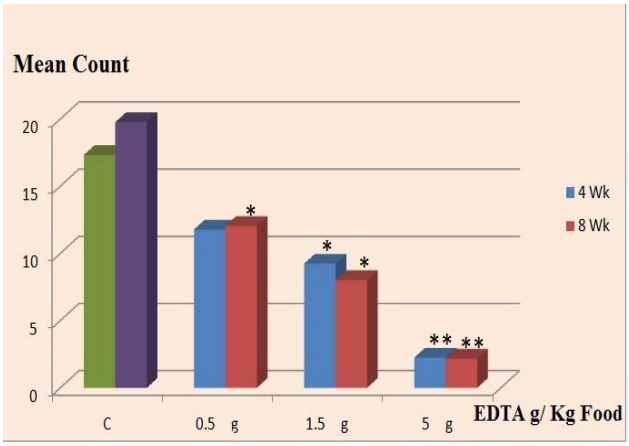

Fig. 6: Percentage of change in sperm count in control and different treated groups.

*** = Significance, **= highly significant.**

Sperm count and morphology

Different numerical sperm values of the different EDTA manipulated mice groups after four and eight weeks experimental periods were shown in (Fig7). Different sperm countswere expressed as X 10^6/ml of semen.

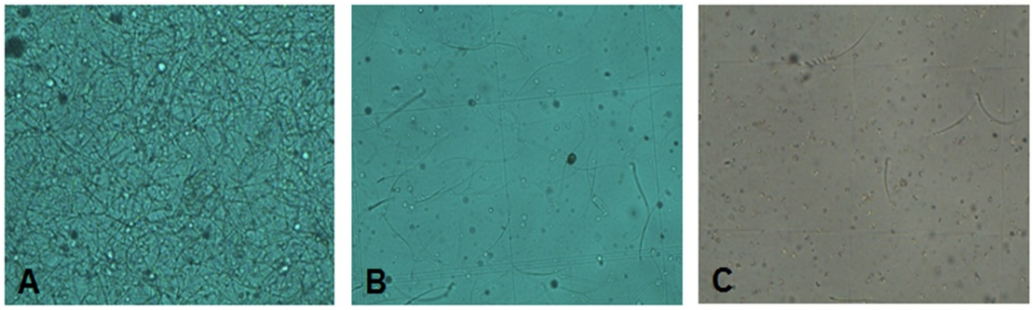

Fig7: Photomicrograph of semenrepresented three different sperm counts throughout the different experimental groups showing the numerical sperm values. A) High sperm count about as over 20X10^6/ml semen; B) moderate sperm count about 2-3X10^6/ml semen and C) low sperm count about 0. 2- 0.3X10^6/ml semen. (100X)

Histological and Histopathological Studies

Control group

Histological observation of testis in control group showed normal testicular structure where each seminiferous tubule is lined with a germinal epithelium and several rows of the successive stages of spermatogenesis: 1-spermatogonia were laying adjacent to the basal lamina and characterized by round to oval nucleus; 2-primary spermatocytes were the largest distinguished by large round nuclei with dark stain; 3-secondary spermatocytes were smaller than the primary spermatocytes and less dense chromatin granules as compared to those of the primary ones; 4-spermatids were the smallest spermatogonic cells with small lightly stained nuclei and 5-spermatozoa appeared with their deeply stained heads and elongated tail and supporting cells of Sertoliscattered in between. They were elongated with large round or oval nuclei. The seminiferous tubules were closely backed with each other leaning triangular space occupied by interstitial cells of Leydgeand blood vessels (Fig8).

EDTA manipulated groups

Testicular tissues of mice received EDTA in the different groups showed numerous differences proportional with the dose of EDTA in comparing with control mice. The group of mice received EDTA (0.5g/kg of food) revealed some necrotic areas in between the germ cells after four weeks that increased through the layer of spermatogonia with appearance of karyolysis after eight weeks.

The interstitial cells possessed dilated and congested blood vessels (Figs. 8B and 9B). The mice exposed to (1.5g/kg of food) of EDTA showed decrement of spermatogenic linage and some degenerated interstitial cells after four weeks while the same group exhibited slug off the spermatogenic cells from the basement membrane with severe degeneration after eight weeks (Figs. 8Cand 9C). The group of mice received the highest dose of EDTA (5.0g/kg of food) showed increased germ cells degeneration, some vacuolated primary cells and the interstitial cells possessed necrotic areas and karyolysis after four weeks in comparison with control. After eight weeks of exposure, the testicular tissue of the same group appeared with severe necrosis in both germ cells and interstitial cells, and foamy appearance of lumen in comparison with control (Figs. 8D and 9D).

The present study exhibited obvious decrement of tunica albuginea thickness proportional with the increase of the dose of EDTA exposure in comparison with control group after eight weeks (Figs . 10 A,B,C and D).

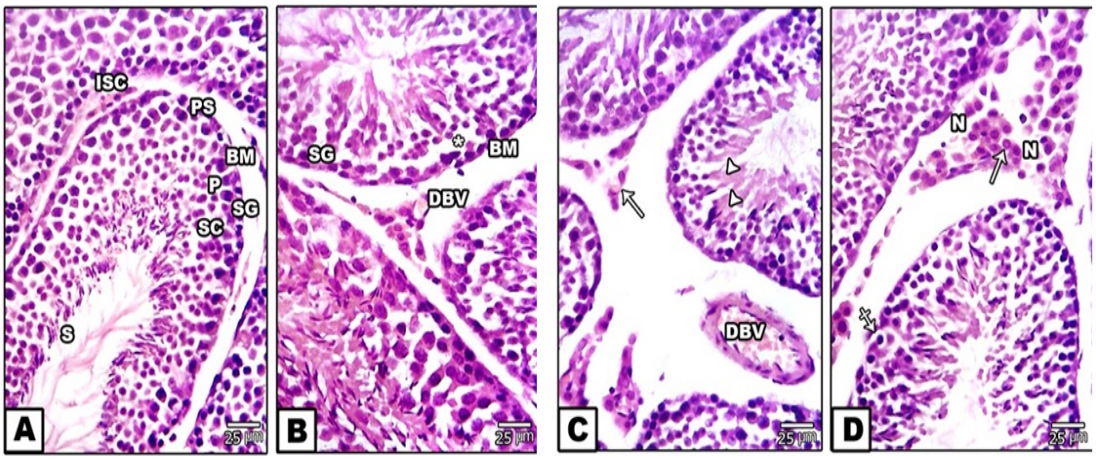

Fig.8:Photomicrographof testis section stained with H & E after four weeks of(A) control group; (B,C and D) three groups of mice manipulated EDTA (0.5g/kg of food), (1.5g/kg of food) and (5g/kg of food) respectively showing:A)normal testicular structures; (ISC) normal interstitial cells; (SC) Sertoli cell. B): (CBV) congested blood vessel through the interstitial cells; (N) necrotic area; (vacuoles in between germ cells); (arrow) less number of spermatozoa. C): (SP) cloudy swelling primary spermatocyte; (arrow) swollen interstitial cell. D): (N) necrotic area through interstitial cells; (arrow) karyolysis; (thick arrow) vacuolated primary spermatocyte.

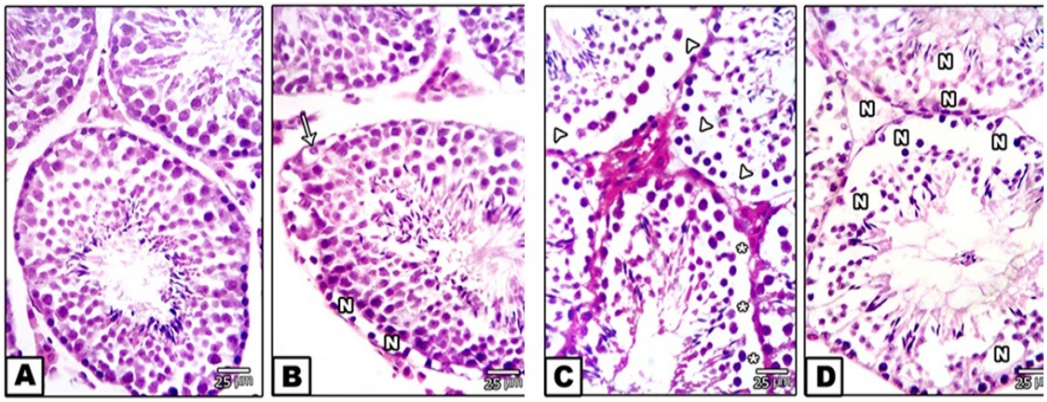

Fig.9:Photomicrographof testis section stained with H&E after eight weeks of(A) control group; (B,C and D) three groups of mice manipulated EDTA (0.5g/kg of food), (1.5g/kg of food) and (5g/kg of food), respectively showing:A)normal spermatogenic structures B): (N) necrotic area at spermatogonia layer; (arrow) karyolysis. C):(astros)slug off the spermatogenic cells from the basement membrane;(arrow heads)degeneration of spermatogenic cells.D):(N)necrotic areas through testicular tissues and interstitial cells;foamy appearance of lumen.

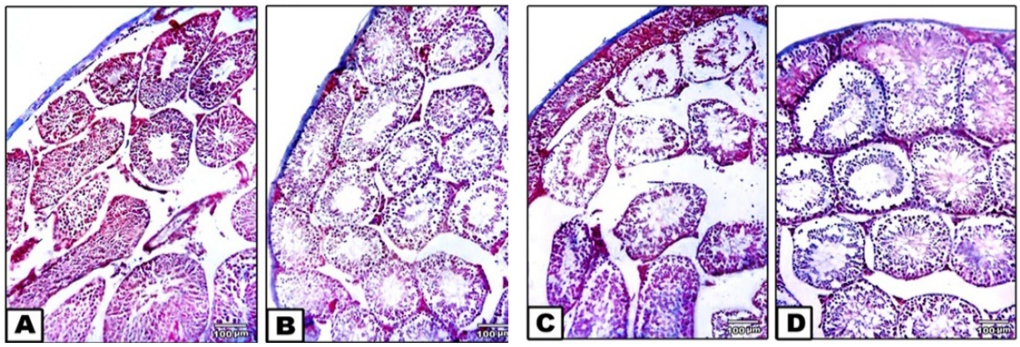

Fig. 10: Photomicrograph of testis section showing Tunica albugineathicknessstained with MT of control and different EDTA manipulated groups after eight weeks.

DISCUSSION

The present study paied attention to evaluate the effect of EDTA mixed with food on the fertility of male mice Now a days, humans have been exposed to different types of toxics that could be effect on the reproduction. The chemical potential for both sexes depending upon the dose and duration of exposure. The adverse effects of various contaminants could be vary from alteration of fertility to induction of cancer (Sedha*et al*, 2015).

Spermatogenesis is a highly organized process of cell proliferation and differentiation leads to mature spermatozoa formation. Exposure to several chemicals and radiotherapy had possible toxic effect on the spermatogenesis (El-kott, 2015).

EDTA is considered as a chelating agent. In medicine, it used for the removal of toxic heavy metals. The disodium salt of EDTA is a common component in eye drops and cleaning solutions. EDTA has been approved by the Food and Drug Administration as a food additive generally recognized as safe(Shackleford, 2004).EDTA potentiates the mutagenic activity of chemical and physical agents in human and mice. It is also cytotoxic and weakly genotoxic, but not carcinogenic. Oral exposures to EDTA produced adverse reproductive and developmental effects in animals (Khalil*et al*., 2008).

In the present study, disodium EDTA as appetizer enhances mice to consume food which had an obvious reflect on the body weight gain as seen in the present datato increase the body weight gain significantly in the highest dose group of exposure. But in contrast with the present study of Yamaguchi *et al*. (1993a; 1993b) mentioned that in trapper it on ealdaily (i.p.) doses of 0.1,0.2, 0.3, 0.5, 1.0 g/kg Calcium Disodium EDTA five days per week for 14 weeks to Wistar rats (6/dose) produced a graded weight reduction. Also, Swenerton and Hurley (1971) reported that, in pregnant rats fed on 2-3% disodium EDTA, with and without zinc supplementation had moderate to severe diarrhea.

In the present morphomertric study to assessment the testis function, primary spermatocytes count of the different EDTA manipulated groups recorded asignificant($P< 0.01$) decrement of the lowest dose of EDTA group proportional with concentration of EDTA and duration time which recorded significant($P< 0.001$) decrement in comparison with control group. The interpretation of the present study suggested that EDTA was toxic for meiotic cells proliferation even at low concentration, and its toxicity increased in a dose-dependent manner.

Kimmel (1975) reported gonad dysgenesis in offspring of pregnant CD rats treated with EDTA on GD 7- 14. EDTA which was administered to the rats by gavage, or by sub cutameousinjection at concentrations of 1000 mg/kg/day, 1250 mg/kg/day, or 325 mg/kg/day, respectively.Feeding of EDTA caused

maternal toxicity, but the females were not died. Seventy-one percent of the offspring had severe malformations similar to those observed by Swenerton and Hurley (1971).

The present data recorded a significant decrease of fertile seminiferous tubules count of the different doses of disodium EDTA manipulated groups in both durations of exposure. Also, the determination of the semen analysis (sperm count, vitality percentage and semen quality) showed significantly decrease that proportional with the increase of EDTA dose manipulation of mice comparing with the control group.

In a feeding study described by Lanigan and Yamarik (2002),four groups of weanling rats (two males and four females per group) were given 0.5-5.0% disodium EDTA. The rats were mated after 100 days. Rats of the high dose group failed to give birth, even though they had been mated for two months. Rats of the untreated control group and those fed 0.5-1.0% of the test chemical had normal first and second birth.

However of Muralidhara and Narasimhamurthy (1991) reported that very low dose of disodium EDTA had no adverse effect on either the testicular or epididymis weights and microscopic structure.The salt (disodium EDTA) was administered in water to Swiss Albino (CFT) mice at doses of 5- 15 mg/kg/day for five consecutive days. Moreover, caudal sperm counts and the incidence of sperm-head abnormalities did not differ from controls. However, EDTA had a synergistic effect with gramicidin (an antiviral and antibiotic) in inhibiting sperm motility and cervical mucus penetration *in vitro (*Bourinbaiar and Lee, 1995).The present investigation interpreted such case that the effect of concentration of EDTA used and the duration period seemed to be responsible for reproductive impairments/or safety in male mice.

In the current histological and histopathological observations, the testicular tissues of mice received the lowest dose of EDTA revealed some necrotic areas in between the germ cells after four weeks that increased through the layer of spermatogonia with appearance of karyolysis after eight weeks in comparing with control mice. The interstitial cells possessed dilated and congested blood vessels.Also mice exposed to the moderate dose of EDTA showed decrement of spermatogenic linage and few degenerated-interstitial cells after four weeks while the same group exhibited slug off the spermatogenic cells from the basement membrane with increased degeneration after eight weeks. In addition, the group of mice received the highest dose of EDTA showed high degree of germ cells degeneration, some vacuolated primary cells and possessed necrotic areas through the interstitial cells after four weeks, and the same group appeared with severe necrosis in both germ cells and interstitial cells after eight weeks of exposure comparing with control.

In a subsequent six-month,Yamaguchi *et al*. (1994) observed focal or total expansion and hemorrhage of the vascular wall, andplexiform-like lesions of the thin-muscular-type arteries,of both sexes of guinea pigs of both they were injected i. p. daily for up to six months with 0.8-1 ml of 6% Disodium EDTA, the animals died of either a rupture of the ventricular wall or of right ventricular failure (Yamaguchi *et al*., 1993a; 1993b).

Kimmel (1975)studied the gonaddysgenesis in offspring of pregnant CD rats that treated with EDTA on GD 7- 14 showing sexual malformed offspring by microscopic examination. The gonads were not well differentiated and the number of germ cells was decreased significantly. Male offspring had small, cryptorchid testicles with few seminiferous tubules; few spermatogonia were observed. The authors reported that the testicular changes were similar to those mentioned in the offspring of zinc-deficient rats and suggested that the observed alterations wereasthe result of the effect of EDTA on the concentration of the endogenous metal.

In the present study, after eight weeks of EDTA manipulation, tunica albuginea thickness showed asignificant decline of the lowest dose of EDTA group while the other two groups of EDTA manipulation exhibited a highly significant decrement in comparison with control group.

From electron microscopy study of Yamaguchi *et al*. (1994a) whoobserved irregularly thickened and fragmented internal elastic lamellae of the pulmonary artery. Was observed the internal elastic lamellae exhibited severe coiling and vacuolar formation was observed in the smooth muscle cells. The lamellae were barely stained by tannic acid, a change suggestive of degenerative change in the elastic components. Focal or total expansion and hemorrhage of the vascular wall, as well as plexiform-like lesions of the thin-muscular-type arteries, were also observed. In a subsequent six-month study dilatation of the left ventricle with a moderately thin ventricular wall, as well as right ventricular changes, were observed. These adverse alterations were concluded due to EDTA-induced calcium deficiency. The low calcium content "loosened" the molecular structure of glycosaminoglycans and glycoproteins, which form important components of the intercellular matrix and elastic lamellae. This change in the components' viscous characters accelerated the detachment of endothelial cells, but did not adversely affect the joining of endothelial cells (Braide, 1984; Yamaguchi *et al*., 1994b).

In conclusion, the present histological results revealed obvious delaying on the spermatogenesis process and degenerated-interstitial cells in mice that proportional with the dose of EDTA and exposure period. Also, the determination of the semen analysis, primary spermatocytes count and fertile seminiferous tubulescountrecorded significantly decreasedin proportional with the increase of EDTA dose manipulation and duration time of mice comparing with the control mice. Based on these results, this study suggested that eating foods mixed with EDTA in different doses used in the current research seems to have a negative impact on the fertility of semen in mice.

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تقييم تأثير الطعام الملوث ب (إ.د.ت.ا) علي الخصوبة في ذكور الفئران

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تهدف هذه الدراسةالي تقييم تأثير الطعام الملوث ب (إ.د.ت.ا) علي الخصوبة في الفئران. حيث يشمل هذا التقييم من عدة متغيرات هي عدد الحيوانات المنويه وقياس حيويتها وكذلك الشكل لها.هذا بالاضافة الي دراسه التغيرات النسيجية والنسيجية المرضية في النسيج الخصوي.ولقد استخدم لهذه الدراسة اربعين فأرا في حالة صحية جيدة وقد تم تقسيمهم الي أربعه مجاميع. المجموعة الأولي وهي المجموعة الضابطة والتي تتغذي علي الأكل الطبيعي للفئران،المجموعه الثانية الي الرابعة فقد تم خلط الطعام اليومي لها بتركيزات مختلفة من ماده (إ.د.ت.ا) وهي (0.5,1.5, و5 جرام /كيلوجرام من الطعام). وقد تم تجميع العينات من كل مجموعة علي فترتين زمنيتين وهما 4و8اسابيع من بدايه خلط الطعام (إ.د.ت.ا). وقد اظهرت النتائج ان المجموعات المعاملة ب (إ.د.ت.ا) توضح تدهورا واضحا ذو دلاله احصائية في جميع المتغيرات المقاسة في تقييم كفاءةالحيوانات المنويه بما تحتويه من حيوانات منوي.وقد اظهرت النتائج كذلك بأن المجموعات المعاملة با (إ.د.ت.ا) توضح تدهورا ملحوظا وذو دلاله احصائيه في جميع المتغيرات المقاسة لعددالخلايا المنوية الإبتدائية وعدد الانبيبات المنوية الخصبةفقد تناقص عددها وذلك بالمقارنة مع المجموعة الضابطة.هذابالإضافة إلي دراسة التغيرات النسيجيةالخصوية فقد وجد ان عدد الخلايا مولدات المني قد قل بنسبة كبيرة وكذلك الخلايا المنوية الإبتدائيه المكونة لها، تليف النسيج الخصوي،احتقان في الاوعيه الدمويه كما لوحظ ايضا انتفاخ في بعض الخلايا المنويه الإبتدائية وتحلل في خلايا سرتولي وكذلك الخلايا البينيةللانبيبات المنوية تناسبت طرديا مع زيادة الجرعة المتناولة من (إ.د.ت.ا) ومدة التعرض لها.من هذه الدراسة نخلص إلي أن التأثير السلبي لتناول الطعام المخلوط ب (إ.د.ت.ا) يحفز علي تناقص نشاط التكوين المنوي لخلايا الخصية والذي يؤدي الي قلة الخصوبة في ذكور الفئران ،والمحصلة من هذه الدراسة تدل علي ان التعرض المزمن لمادة (إ.د.ت.ا)بتناولها في الطعام وبنسب عالية يؤدي الي اضرار بالخصية والذي تم اثباتة من خلال القياسات المورفومترية وكذلك النسيجية المرضية.