EFFECT OF VITAMIN E INJECTION ON RATS INDUCED WITH BREAST CANCER AND THEIR RELATION TO BONE DISTURBANCE

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ABSTRACT

The present study was carried out to study the inhibitory effect of vitamin E on breast cancer induced by N-methyl-N-nitrosourea (MNU) in female Sprague-Dawley rats. The effect of vitamin E on serum levels of tumor marker Carcinoembryonic antigen (CEA), estrogen, progesterone hormones, alkaline phosphatase (ALP) activity, red blood cells (RBCs) count, white blood cells (WBCs) count and hemoglobin were determined. In additional calcium, inorganic phosphates, femoral thickness, femoral length, breaking force, femoral wet weight, bone volume and bone density were evaluated to study the effects of vitamin E on bone. The results showed that the serum progesterone, hemoglobin levels and RBCs count were significantly higher (p<0.05) in the treated group than in the breast cancer group. Significant lower (p<0.05) was noticed in the CEA, estrogen, WBCs and ALP activity in treated group than in breast cancer group. There was significant increase in calcium, phosphate, femoral length, breaking force, femoral wet weight, bone volume and bone density in treated group than in breast cancer group. There was significant increase in calcium, phosphate, femoral length, breaking force, femoral wet weight, bone volume and bone density in treated group than in breast cancer group. The increase levels of these parameters with time and the decrease in CEA, estrogen, WBCs and ALP activity shows that they could be of importance in monitoring cancer treatment and disease progress in a resource-poor setting. Histopathological analysis was carried out to confirm the efficacy of vitamin E in the inhibition of breast cancer induction in female Sprague-Dawley rats.
Key words: Breast cancer, progesterone, Estrogen, Bone disturbance, Vitamin E

INTRODUCTION

Breast cancer is the most common type of cancer and the second leading cause of cancer death in American women. In 2002, 209,995 new cases of breast cancer were registered, and 42,913 patients died of it. In 5 years, the annual prevalence of breast cancer will reach 968,731 cases in the United States. Worldwide, the problem is just as significant, as breast cancer is the most frequent cancer after nonmelanoma skin cancer, with more than 1 million new cases in 2002 and an expected annual prevalence of more than 4.4 million in 5 years. Breast cancer treatment currently requires the joint efforts of a multidisciplinary team (Gonzalez-Angulo et al., 2007).

Antioxidants such as vitamins A, C, E, and selenium, have drawn a lot of attention to the scientists and the public alike (Borek, 2004). They have been shown in experimental studies to neutralize or trap reactive oxygen species (ROS), thereby preventing cellular damage caused by the reaction of these species with proteins and nucleic acids (Borek, 2005 and Thomson et al., 2007).

Although the biological effects of vitamin E have been investigated over many decades, its role in inhibiting breast carcinogenesis remains incomplete. The aim of this study was to evaluate the effect of vitamin E on breast tumor growth. The effect of vitamin E on plasma tumor marker (CEA), and some biochemical parameters as well as bone density, breaking force, thickness and length of femur of rats were evaluated in order to accomplish the anticancer effect of this vitamin.

MATERIALS AND METHODS

1. Materials:
Chemicals:

Vitamin E, N-methyl-N-nitrosourea (MNU) were obtained from Sigma Chemical Company, USA, and all chemicals used throughout the whole work were of analytical grade.
Reagent methodology:
Carcinoembryonic Antigen (CEA) kit was purchased from Pishtaz Teb Diagnostics Ltd. Korbach/Germany. Alkaline phosphatase (ALP) kit was obtained from DiaSys Diagnostic Systems, Germany. Progesterone and estrogen kits were purchased from BioCheck, Inc. Foster City, USA.

Experimental animals:
The female Sprague-Dawley rats, with an average weight of 110-120 g were used in this study. The animals were obtained from Holding Company of Biological Sera and Vaccines (VACSERA), Cairo, Egypt.

2. Methods:

Experimental animals:
Thirty female Sprague-Dawley rats were used in this study. After the adaptation period, the rats were classified into three groups as follows: Group (1): rats were intraperitoneally injected with physiological saline two times per week for 6 months and worked as negative control. Groups 2 and 3: rats were intraperitoneally injected with single dose of MNU (50 mg/kg body weight) to induce breast cancer in the mammary glands of rats. After appearance of breast cancer rats of Group 3 were intraperitoneally injected with vitamin E [75mg/kg body weight] twice a week for 6 months. At the end of the experimental period (6 months), rats were anesthetized with diethyl ether and blood specimens were withdrawn from the retro-orbital plexus using capillary sterile glass tube and collected to get sera. After that, rats were killed by decapitation and mammary tumors and right femur bone were quickly removed. The right femur used for measurements of bone, while mammary tumors were examined histopathologically.

Biochemical parameter of serum:

Determination of tumor marker CEA:
Serum CEA was determined using Pishtaz Teb Diagnostics Ltd. Korbach/Germany according to Goldenberg et al. (1981).

Determination of serum estrogen and progesterone:
Serum estrogen and progesterone were determined using BioCheck, Inc. Foster City, USA according to Siiteri et al. (1982).
Determination of serum alkaline phosphatase (ALP) activity:
Serum ALP activity was determined according to the colorimetric method (Ross et al., 1981) using DiaSys Diagnostic Systems, Germany.

Analysis of hematology parameters:
Red blood cells (RBCs), Hemoglobin (Hb) and white blood cells (WBCs) were analyzed by Automated Blood Counter Veterinary (ABC Vet.) User Manual, RAB 015 A Ind. A, France.

Physical measurements of rat femur bone:
Measurement of rat femur length and thickness:
The length and thickness of the femur were measured with a dial caliper according to Moss et al. (1999).

Measurement of femoral wet weight, bone volume and bone density:
The wet weight, bone volume and bone density of the femur were measured according to Iwamoto et al. (2004).

Measurement of rat femur breaking force:
The breaking force of the femur was measured according to Tamaki et al. (2003) using the Digital Force Gauge (Model FGN-50, Japan).

Determination of femoral calcium and total phosphorous:
The femurs (g) were oven dried at 105 °C for constant weight. The dried bones were ashed in a muffle furnace at 600 °C for 6 hours. Calcium and total phosphorous of the femurs ash were measured according to Potter et al. (1995).

Histopathological examination:
Histopathological studies were carried out according to Banchroft et al. (1996).

3. Statistical analysis:
The collected data from four repetitions of any experiment were statistically analyzed in triplicate. Data were presented as mean values±SD. Data were analyzed to one-way analysis of variance (ANOVA) and least significant difference (LSD) at p<0.05 followed by Duncan's new multiple range tests to assess differences between group's means (Waller and Duncan, 1969).
RESULTS AND DISCUSSION

1. CEA tumor marker:

The effect of 24 weeks treatment with vitamin E on serum concentration of CEA was determined in all groups of rats. The results in table (1) show that the concentration of CEA was increased in breast cancer group (8.027ng/ml) than in negative control (2.85ng/ml) by 181%. The treatment with vitamin E significantly reduces the elevation of CEA (5.9 ng/ml) in animals with cancer by 26 %. Persistently rising CEA value may be associated with progressive malignant disease and a poor therapeutic response. A declining CEA value is generally indicative of a favorable prognosis and a good response to treatment. Patients who have low pre therapy CEA levels may later show elevations in the CEA level as an indication of progressive disease. Clinical relevance of the CEA assay has been shown in the follow-up management of patients with colorectal, breast, lung, prostatic, pancreatic, and ovarian carcinoma (Malati, 2007).

2. Hormone analysis:

Serum concentration of estrogen and progesterone was determined in different groups and the data are given in Table (1). The results show that the concentration of estrogen hormone was increased in breast cancer group than in negative control by 144%. The treatment with vitamin E reduced this percentage to 108%. The results in the table also show that the concentration of progesterone decreased from 9.62 Pg/ml in negative control to 2.59 Pg/ml in breast cancer group (271 %). The treatment with vitamin E increased the level of progesterone hormone to only 7.61 Pg/ml instead of 2.59 Pg/ml in breast cancer group. The response of cells to estrogen depends on whether they have estrogen receptors. Classifying breast cancers by their estrogen receptor status (estrogen receptor positive (ER+) or estrogen receptor negative (ER-)) is done to assist in the selection of appropriate therapies some ER+ cancers respond favorably to hormone blockers while ER- cancers do not. Several studies have found that ER+ and ER- breast cancers have distinctly different risk factors and therefore, possibly different etiologies (Meiners, 2011). In general, ER+ breast cancers are more commonly correlated with reproductive related risk factors associated with endogenous estrogen
EFFECT OF VITAMIN E INJECTION ON RATS INDUCED EXPOSURE, SUCH AS EARLY MENARCHE, NUMBER OF PREGNANCIES, AND LATE AGE CHILDBEARING (Althuis et al., 2004 and Meiners, 2011). Estrogens have been implicated in breast cancer; however, the mechanism of action still remains unclear. One theory suggests that the mechanism is dependent on the activation of the ER. Estrogen induces breast cancer through stimulation of cellular proliferation, resulting in more opportunities for accumulation of genetic damages leading to carcinogenesis (St-Hilaire et al., 2011). Another possible mechanism of action may be through the metabolism of estrogen, which may induce oxidative stress and play a key role in mammary cancer development (Russo et al., 2003 and Mense et al., 2008). Estrogen metabolites may exert DNA mutations from ROS or DNA mutations which may lead to the accumulation of genomic alterations essential for mammary tumorigenesis (St-Hilaire et al., 2011).

Table (1) Serum biochemical parameters of breast cancer induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>CEA conc.(ng/ml)</th>
<th>Estrogen conc. (Pg/ml)</th>
<th>Progesterone conc. (ng/ml)</th>
<th>ALP activity (U/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>2.85±0.129</td>
<td>14.15±0.575</td>
<td>9.6225±0.1967</td>
<td>277.25±9.639</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>8.07±0.166</td>
<td>34.51±0.810</td>
<td>7.50±0.1095</td>
<td>473.25±13.865</td>
</tr>
<tr>
<td>Vit. E + breast cancer</td>
<td>5.9±0.183</td>
<td>29.51±0.889</td>
<td>7.615±0.2359</td>
<td>378.5±10.116</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviation. Within the same column, various superscript letters indicate significant differences (Duncan, P <0.05).
ALP: Alkaline phosphatase, CEA: Carcinoembryonic antigen

3. Alkaline phosphatase activity (ALP):

Serum ALP activity was significantly higher (p<0.05) in breast cancer induced by MNU (473.25 U/l) than in the negative control group (277.25 U/l) by 70.7 % (Table 1). By treatment with vitamin E the ALP activity was decreased in breast cancer group by 20 % compared with breast cancer group. Women with breast cancer have higher calcium levels and higher alkaline phosphatase (ALP) and acid phosphatase (ACP) activities than normal healthy women (Usoro et al., 2010). The increased activity of this enzyme was noticed in subjects who may also be due to osteolytic bone metastases in breast
cancer leading to increased osteoclastic activity and bone resorption. Women with breast cancer have higher ALP activity than normal healthy women. The progressive increase in the serum ALP activity during the six months follow up is an indication that measurement of this parameter may be useful tools in monitoring treatment and disease progression in areas where facilities for sophisticated studies are not readily available (Fishman et al., 1968).

Serum of breast cancer patients has higher calcium levels and higher ALP and ACP activities. The increase in the levels of these parameters with time shows that they could be of importance in monitoring treatment and disease progress in a resource-poor setting (Keshaviah et al., 2007).

4. Hematology parameters:

Hemoglobin (Hb), Red blood cells (RBCs), and white blood cells (WBCs) were analyzed in all groups and the data are given in Table (2). The results show that there is a significant change in the concentration of hemoglobin and number of RBCs which were decreased in cancer induced group (7.49g/dl, 7.07 x 10^6, respectively), compared with negative control (15.83g/dl, 8.52 x 10^6, respectively). The treatment of cancer induced animals with vitamin E increased both hemoglobin concentration and RBCs number (13.58 g/l, 8.05 x 10^6 mm3) when compared to cancer induced group (7.49g/dl, 7.07 x 10^6, respectively). Significant elevation in the number of WBCs in cancer induced group (16755 /mm^3) compared with negative control (8275mm^3). The treatment with vitamin E improved the WRBs number which reached to 13725 / mm^3. These results are in agreement with Usoro et al. (2010) who reported that mean baseline hemoglobin level in breast cancer patients was 10.5 ± 0.9 (SD) g/dL. RBC counts were reduced from 7% to 2% in the breast cancer population. In group with breast cancer, the injection of vitamin E at 75mg/kg twice/week increased the concentration of hemoglobin and RBCs count. Blood hemoglobin concentration seems to affect the prognosis of patients with early breast cancer when a treatment schedule that includes radiotherapy is applied (Blayney et al., 2003).
Table (2) Hematological parameters of breast cancer induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Hb (g/dl)</th>
<th>RBCs/ mm3</th>
<th>WBCs/ mm3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>15.83±0.7395a</td>
<td>(8.525×10^6±0.263×10^6)a</td>
<td>8275±275.3785c</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>7.4925±0.4549c</td>
<td>(7.075×10^6±0.2217×10^6)c</td>
<td>16575±434.9329d</td>
</tr>
<tr>
<td>Vit. E + breast cancer</td>
<td>13.58±0.6229b</td>
<td>(8.05×10^6±0.1291×10^6)b</td>
<td>13725±386.2210b</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviation. Within the same column, various superscript letters indicate significant differences (Duncan, P <0.05).

Hb: hemoglobin, RBCs: red blood Cells, WBCs: white blood cells

5. Calcium and total phosphorous contents:

The data in table (3) showed that Ca and P were significantly lower (p>0.05) in femur bone of breast cancer rats than in negative control by 18%, 17%, respectively. The treatment with vitamin E recurrence these failure of calcium and phosphate concentrations by 10%, 9 %, respectively. These results show that femur bone of rats with induced breast cancer have lower total calcium and phosphorous levels than in the negative control group. The decrease of Ca and P in the bone is resulting from demineralization of bone. A decrease in ionized calcium stimulates a release of parathyroid hormone (PTH), which maintains calcium homeostasis by increasing bone mineral dissolution, thus releasing calcium and phosphorus, increasing renal reabsorption of calcium and excretion of phosphorus, and enhancing the gastrointestinal absorption of both calcium and phosphorus indirectly through its effects on the synthesis of 1,25(OH)2D (calcitriol). In healthy subjects, this increase in serum PTH level in response to hypocalcemia effectively restores serum calcium levels and maintains normal serum phosphorus levels (Moe, 2008).

Table (3) Femur bone calcium and phosphorus contents of breast cancer induced rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium Conc. (%)</th>
<th>Phosphorus Conc. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>16.62±0.3509a</td>
<td>7.3477±0.2742a</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>13.5533±0.3661c</td>
<td>5.9047±0.2002c</td>
</tr>
<tr>
<td>Vitamin E + breast cancer</td>
<td>14.8767±0.3774b</td>
<td>6.4487±0.2728b</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviation. Within the same column, various superscript letters indicate significant differences (Duncan, P <0.05).
6. Physical measurement of rat femur bone:

Femoral thickness, femoral length, breaking force, femoral wet weight, bone volume and bone density were determined in different groups and the data are given in Table (4). The results show that rats injected with MNU (breast cancer group) reduced maturation-related gains in femoral thickness, femoral length, breaking force, femoral wet weight, bone volume and bone density by 5.95, 9.86, 28.4, 32.4, 10.97 and 24.1%, respectively when compared to negative control group. The treatment with vitamin E caused a significant increase in femoral length, breaking force, femoral wet weight, bone volume and bone density by 3.59, 10.78, 28.27, 11.59 and 15.0%, respectively compared with breast cancer group. Whereas, no significant change in femoral thickness was noticed. The data presented in the present study clearly demonstrate that a dose of 75mg/kg/ twice week of vitamin E prevent bone loss. Similar results were obtained by Bogden et al. (2008) who mentioned that vitamin E prevents loss of bone by reduce estrogen synthesis. The hormonal activity in the case of male and female rats plays an important role in their physicochemical development that includes muscle and bones. In female rats, estrogen is one of the major regulating factors responsible for bone formation and maintenance and the loss of this hormone after menopause often leads to bone loss in the case of human (McMillan et al., 2007).

Table (4) Physical parameters of femoral bone of breast cancer induced rats

<table>
<thead>
<tr>
<th>Parameter / Group</th>
<th>Negative control</th>
<th>Breast cancer</th>
<th>Vit. E+ Breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral thickness (mm)</td>
<td>2.645±0.0420a</td>
<td>2.4875±0.0562b</td>
<td>2.52±0.0469b</td>
</tr>
<tr>
<td>Femoral length (mm)</td>
<td>33.2±0.4243a</td>
<td>29.925±0.4573c</td>
<td>31±0.5715b</td>
</tr>
<tr>
<td>Breaking Force (N)</td>
<td>90.595±1.1301a</td>
<td>64.79±1.9657c</td>
<td>71.7725±1.2856b</td>
</tr>
<tr>
<td>Weight of bone (g)</td>
<td>0.6238±0.021a</td>
<td>0.4216±0.0169c</td>
<td>0.5408±0.0091b</td>
</tr>
<tr>
<td>Bone volume (ml)</td>
<td>0.3875±0.0126a</td>
<td>0.345±0.0058b</td>
<td>0.385±0.0129a</td>
</tr>
<tr>
<td>Bone density (g/ml)</td>
<td>1.61±0.0324a</td>
<td>1.2218±0.0319e</td>
<td>1.4055±0.0313b</td>
</tr>
</tbody>
</table>

All values are expressed as mean ± standard deviation. Within the same level, various superscript letters indicate significant differences (Duncan, P <0.05).
7. Histopathology

Since the histological structure of rats mammary glands tumors resembles those of human ones (Thompson et al., 1995). The induction of mammary carcinomas by the application of MNU to female rats is one of the most frequently used animal models for the investigation of breast carcinogenesis and mammary tumors treatment. This experiment study was designed to evaluate the efficacy of vitamin E in the inhibition of MNU induced breast cancer in female rats. Fig (1A) shows that no histopathological alterations were observed in mammary gland of control group. There is no histopathological alteration detected in regional lymph in control group (Fig. 1B). Mammary glands tumor was observed at 10 weeks after MNU injection in 65% of rats which indicated that the MNU has strong tumorigenicity properties. Rats injected with MNU, had growth lesions, most of the lesions were tumoral and among them, the vast majority were carcinoma. Tissue masses were originated from mammary glands associated with the ulceration and laceration of the outer skin surface at the area of the teat (Fig. 2).

The histopathological features tumor developed in rats injected by MNU were anaplastic activity in the lining epithelium associated with cystic dilatation in some of them as well as inflammatory cells infiltration in stromal connective tissue and categorized under adenocarcinoma (Fig. 3 A, B). In adenocarcinoma, malignant tumors are derived glandular epithelium and have an adenomatous appearance. The epithelium cells are arranged in gland-like structures surrounding a lumen. Nucleoli are often prominent and mitotic figures are abundant. The mammary glands had anaplastic acini forming group of neoplastic cells associated with irregular proliferation of fibroblastic cells bundles which were categorized under fibro adenocarcinoma (Fig. 4). The regional lymph node of MNU treated rats showed sever congestion in the blood vessels with lymphoid depletion (Fig. 5). Treatment with vitamin E leads to reduction in the number of the rats having the tumor as well as in the decrease of the size of the tumor (Fig. 6). Moreover, there was a great change in the type of tumor after treatment of vitamin E which proved in complete absence of malignancy. As seen in (Fig. 7) mammary gland of rats treated with vitamin E showed the proliferation of fibrous tissues running deferent direction (fibroma) (benign tumor). Lymph gland in regional area of mammary gland of rats treated with vitamin E showing normal histological structure of the lymphoid follicles.
Fig. (1) (A) Mammary gland of rat in control group showing normal histological structure of the acini (a) and ducts (d). (B) Lymph gland in regional area of mammary gland of rat in control group showing normal histological structure of the lymphoid follicles (m). H&E X 80.

Fig. (2) Mammary tumors were observed in MNU treated group. Tumors grew rapidly with irregular and lobulated appearance (A). Some teats suffered ulceration and laceration at the skin surface (B).
Fig. (3) (A) Mammary gland of rat in MNU group showing anaplastic acini as well as cystic acini with inflammatory reaction (adenocarcinoma) H&E X16. (B) Mammary gland of rat in MNU group showing (adenocarcinoma) H&E X40.

Fig. (4) Mammary gland of rat in MNU group showing group of neoplastic cells replacing the mammary acini associated with irregular proliferation of fibroblastic cells (Fibro adenocarcinoma). H&E X 40.

Fig. (5) The regional lymph gland (ph) of rats in MNU (breast cancer group) showed severe congestion in the blood vessels (v) with lymphoid depletion H&E X 60.
Conclusions:

As Conclusion data presented here clearly demonstrate that intraperitoneal injection of 75mg/vitamin E twice /week for 6 months was associated with statistically significant reduction in breast cancer group induced by MNU. This was appeared in the decrease in the concentrations of tumor marker (CEA), progesterone hormone, serum alkaline phosphatase activity. Also, the vitamin treatment into the cancer induced animals improved the severe changes in the
concentration of hemoglobin, RBCs count and WBCs count after treatment with vitamin E. The data presented here also clearly demonstrate that vitamin E prevents bone loss which appeared in decrease in estrogen hormone and increase of bone calcium and phosphate. In addition, Vitamin E recurrence the failure of physical properties of bone by increase the weight, density and breaking force of femoral bone of cancer induced rats. The encouraging results obtained from this work represent an important step towards using natural compounds as inhibitory substances against breast cancer. This vitamin could be as a source for new lead structures in drug design to combat cancer and natural antioxidants. Further investigations are needed to confirm our results.

REFERENCES


تأثر حقن فيتامين هـ على الجرذان المصابة بسرطان الثدي وعلاقته بالعظام

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أجري هذا البحث لدراسة التأثير المشتت فيتامين هـ على سرطان الثدي المستحدث في
إناث الجرذان بمادة الميثيل - نيتروزو بوريا. تم دراسة تأثير فيتامين هـ على مستويات
علامات الورم (CEA)، هرمونات الإستروجين والبروجسترون، نشاط أنزيم الألكالين
فوسفاتيز، أعداد كرات الدم الحمراء والبيضاء والهيموغلوبين. بالإضافة إلى الكالسيوم،
الفوسفات الغير عضوي، سمك، طول، قوة الكسر، الوزن الرطب بثافة العظام، حجم وكثافة
عظام الفخذ وذلك لتقييم تأثير فيتامين هـ على العظام. وأظهرت النتائج أن هناك زيادة معنوية
في هرمون البروجسترون، الهيموغلوبين، أعداد كرات الدم الحمراء للمجموعة
التي تلقت العلاج مقارنة بالمجموعة المصابة بسرطان الثدي. كما لوحظ انخفاض معنوي
(P<0.05) في هرمون الاستروجين، كرات الدم البيضاء ونشاط الألكالين فوسفاتيز
في المجموعة التي تلقت العلاج مقارنة بالمجموعة المصابة بسرطان الثدي. كما كان هناك
زيادة معنوية في الكالسيوم والفوسفات، كثافة العظام، قوة الكسر وطول عظم الفخذ في
المجموعة التي تلقت العلاج مقارنة بالمجموعة المصابة بسرطان الثدي. ارتفاع مستويات هذه
التحاليل مع مرور الوقت والانخفاض في CEA، هرمون الاستروجين، كرات الدم البيضاء
ونشاط الألكالين فوسفاتيز يوضح أهمية ذلك في علاج السرطان. كما أجري تشريح لأنسجة
الورم (الهستوبيولوجي) لتأكيد فعالية فيتامين هـ في تثبيط سرطان الثدي في إناث الجرذان.