Some Immunuological Effects of Danazol on Female Albino Mice

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الخلاصة

درست التأثيرات المناعية للدانازول على اناث الفئران البيض من خلال توظيب المعايير التالية : عدد خلايا الدم البيض (الكلي والتفريقي) ومعامل الانقسام الخيطي (نقي العظم والطحال) والفعالية البلعمية (خلايا غشاء الخلب). استعملت أربعة جرع من هذا العقار (٢٠٠، ٤٠٠، ٦٠٠، ٨٠٠ مايكروغرام/فأره) . انخفض العدد الكلي لخلايا الدم البيض عند الجرعة الاولى (٧٢٥٠خلية/ملم^3 دم)، ومن ثم ارتفع عند الجرعة الثانية (١٠٦٢٥خلية/ملم^3 دم)، إلا انه عاود الانخفاض في الجرعة الثالثة (٩٣٠٠خلية/ملم^3 دم) والجرعة الرابعة (٦٧٢٥خلية/ملم^3 دم) مقارنة بالعدد في حيوانات السيطرة (٧٥٢٥خلية/ملم^3 دم). أما العد التفريقي فقد استجاب على منوال العد الكلي لخلايا الدم البيض، ألا أن خلايا وحيدة النوى أظهرت تغايرا عدديا اكبر. أما معامل الانقسام الخيطي، فقد عززت الجرعتين الوسطى (٤٠٠ و ٦٠٠ مايكروغرام/فأرة) معدلاته في خلايا نقي العظم و الطحال، الا أن الجرعة ٨٠٠ مايكروغرام/فارة خفضت تلك المعدلات (١٨،٤ و ٦،٧ % مقابل ٢٢ و ١٨،٥% على التوالي). أما الفعالية البلعمية فقد تأثرت سلبيا نتيجة المعاملة بالدانازول، وان زيادة الجرعة أدت إلى انخفاض معدلات تلك الفعالية (الجرعة ٨٠٠ مايكروغرام/فأرة: ٧،٣ مقابل ٢ ٠% في السيطرة).

ABSTRACT

The immunological effect of danazol on female albino mice was investigated by employing leukocyte counts (total and differential), mitotic

index (bone marrow and spleen) and phagocytosis (peritoneal macrophages) as parameters for such investigation. Four doses of the drug were used; 200, 400, 600 and 800 µg/mouse. The total leukocyte count showed a reduced level (7250 cells/cu.mm.blood) at the first dose, and then increased at the second dose (l0625 cells/cu.mm.blood). The third does again decreased the count (9300 cells/cu.mm.blood) and further decrease was observed in does IV (6725 cells/cu.mm.blood), compared to a control count of 7525 cells/cu.mm.blood. The differential count responded in a similar manner, but a dramatic fluctuation in the monocyte count was observed. The median doses of danazol (300 and 400 µg/mouse) showed some enhancement of the mitotic activities of bone marrow and spleen cells, while a higher dose (800 µg/mouse) reduced such activity (l8.4 and l6.7% vs. 22.0 and l8.5%, respectively). The phagocytosis was negatively affected by the danazol treatments, and increasing the dose reduced such activity (dose 800 µg\mouse: 7.3 vs. 20.0 % in control).

INTRODUCTION

Danazol is the drug of choice in the treatment of benign breast disease, endometriosis and related endocrine disorders, and hereditary angioedema (l). It is a synthetic hormone derived from ethisteron, with the ability to suppress the pituitary gonadal axis by inhibiting the output of pituitary gonadotrophins in both males and females (2). Therefore, a modification of hormonal level may outcome due to the drug use, especially sex hormones. It is well suggested that sex hormone levels may effect immunological functions (positively or negatively). Such a view is

supported by the employment of danazol therapy to treat hereditary angioederma, which is a disorder with a known immunologic defect (a deficiency in Cl inhibitor) (3). Accordingly, the present study was planned with the aim to evaluate the immunological status of female mice treated with different doses of danazol, by employing leukocyte count (total and differential), phagocytosis and mitotic activity of two lymphoid organs (bone marrow and spleen), as parameters for such evaluation.

MATERIAL AND METHODS

*1- **Animals***: Female albino mice (Balb/c; *Mus musculus*) were the subjects of the study, which were purchased from the Institute of Sera and Vaccines (Baghdad). They were 8-10 weeks old at the time of experiments.

*2- **Danazol***: The Ministry of Health (Iraq) supplied Danol (danazol) capsules (100 mg), and they were products of Sanofi Winthrop Limited, United Kingdom.

*3- **Laboratory investigations***: Leukocyte counts (total and differential) were made by conventional hematological methods in blood obtained from the tale of mouse (4). The Phagocytosis was assessed in peritoneal cells *in vitro*. Briefly, after obtaining the cells, they were washed two times with phosphate buffer saline (PBS) supplemented with 10% heat inactivate foetal calf serum. The cells suspended in Hanks balanced salt solution (Ca^{+2} and mg^{+2} free), counted and adjusted to a concentration of 5×10^6 cells/ml. An aliquot of 0.2 ml cell suspension was incubated with 0.1 ml human serum (AB negative) and 0.2 ml of heat-killed yeast (*Saccharomyces cerevisiae*)

for 15 minutes in a water bath (37 ^{0}C). The mixture then transferred to ice bath, and the phagocytic cells assessed microscopically and expressed as a percentage of the total cells (5). The mitotic activity was assessed in cells obtained from bone marrow and spleen after two hours from injecting the animals (intrapenitoneally) with colchicine (0.25 mg/mouse). The harvested cells were washed, treated with hypotonic KCl (0.075M), fixed in methanol-glacial acetic acid (3:1) and stained with Giemsa dye. The dividing cells were scored microscopically and expressed as a percentage of the total cells (6).

4- Experimental design: The animals were divided into five groups, each with four mice. The first one was a control group (untreated animals). The second, third, fourth and fifth groups were treated orally for six days with four different doses of danazol (200, 400, 600 and 800 µg\mouse, respectively). The dosing regimen was based on the normal course of human therapy.

5- Statistical analysis: The differences between treated and untreated animals were assessed by the student t-test.

RESULTS

In table 1, the distribution of leukocyte counts (total and differential) in untreated (control) and danazol-treated mice is presented. The obtained danazol count in the control mice was 7525±229 cells/cu.mm.blood. Treating the mice with the first dose of danazol (200 µg/mouse) reduced the

count to 7250±202 cells/cu.mm.blood, although the difference was not significant. However, the second treatment (dose: 400 mg/mouse) increased the count to l0625±427 cells/cu.mm.blood. Such difference was highly significant At dose III (600 µg/mouse), the count decreased (9300±216 cells/cu.mm.blood), but it remained higher than the control value. Such decrease was more pronounced in the fourth treatment (800 µg/mouse), and the value was below the control count (6725±240 vs. 7525±229 cells/cu.mm.blood). The difference was also significant

The leukocyte differential count responded in different manner, which was related to the type of leukocytes. The lymphocytes showed a decreased count at dose I (3l0l±l28 cells/cu.mm.blood), increased counts at doses II and III (4057±l34 and 4445±32l cells/cu.mm.blood, respectively) and a decreased count at dose IV (3434±l33 cells/cu.mm.blood) when compared to the control count (3847±213 cells/cu.mm.blood). The deviation in the first dose was only significant. These deviations when considered in term of frequencies, the picture may come up different. The fourth dose, which caused a decreased lymphocyte count, shared a similar percentage with the control group (5l.l%), while the second dose caused the lowest frequency (38.2%). The neutrophils showed increased counts at the first and second doses (3389±97 and 4308±214 cells/cu.mm.blood, respectively), and a decreased count at the fourth dose (2ll9±86 cells/cu.mm.blood). In the third dose, although the count of neutrophils was higher than the control one (3309 ±2l4 vs. 2668±69 cells/cu.mm.blood), it showed a decreased level when compared to the count of second dose. Most of these deviations reached a significant level (Table l). Again, the percentage assessment may contradict these outcomes, and the dose 600µg/mouse shared a similar

frequency with the control group (around 35%). Much more deviation were observed in monocyte count, which showed a significant reduced level in the first dose (580±29 cells/cu.mm.blood), and thenceforth, the count showed a dramatic increase (range: 1157±81 - 1892±126 cells/cu.mm.blood), compared to a control value of 693±19 cells/cu.mm.blood. The third and fourth doses although they showed gradual decreased counts, their monocyte values were still higher than the control count, and the differences were significant. The monocyte percentage maintained a level of increased frequencies in the last three treatments with a range of 17.2 – 19.2%, compared to a frequency of 9.2% in the control group. The eosinophils were badly affected by the danzol treatment, both at the levels of count and percentage, especially at the last two doses 44±14 and 17±7 cells/cu.mm.blood, respectively), when compared to the untreated animals (245±37 cells/cu.mm.blood). The second treatment was an exception, and the eosinophil count increased to 370±53 cells/cu.mm.blood. These deviations were significant. The effect of danazol treatment in the phagocytic activity of mouse peritoneal cells was also evaluated (Table 2). Such activity scored a phagocytic index of 20% in the untreated mice, and this value was highly disturbed by the drug treatment, especially in the last three doses, which showed significant decreased levels (9.1, 9.6 and 7.3%, respectively).

The mitotic index of bone marrow and splenic cells was also affected by the danazol treatment, although the manners were different (Table 2). The bone marrow cells showed a non-significant decreased frequency of mitotic index in the first dose (21.5%) when compared to the controls (22.5%). The

second and third doses maintained increased frequencies (31.5 and 27.3%, respectively), while the fourth treatment reduced such activity to a value of 18.4%. The splenetic cells shared the effect of the fourth dose in the bone marrow cells, and a decreased frequency was observed (16.7%) when compared to the control frequency (18.5%). However, the third dose increased the mitotic index to 25.1%. Some of these differences reached a significant level (Table 2).

DISCUSSION

The presented results demonstrated those danazol treated mice showed deviations (positively or negatively) from normality in the parameters investigated. Such deviations were dose-dependent, moreover, they were interrelated and matching each others with respect to the related sites of investigation in the animals. The blood leucocyte count decreased in the first dose, increased in the second dose, and decreased again in the last two doses. Such fluctuations accounted for 3.7, 44.2, 12.4 and 10.6% of the control value respectively. A close look at the mitotic activity of bone marrow cells (Table 2) shows a similar pattern of deviations at the same doses (2.3, 43.2, 24.1 and 16.4% respectively). Such observations may suggest that the primary action of danazol is on bone marrow stem cells, which then pictured in the peripheral blood. Three previous observations support such view, and danazol has been used successfully in treatment of bone marrow failure in some forms of myeloid metaplasia (7, 8, and 9). In the latter study (9), a 13-year-old girl was encountered with the diagnosis of aplastic anemia due to hypoplastic myelodysplastic syndrome. A combined treatment of cyclosporin A (and other drugs) and danazol made the patient

transfusion independent one month later. Moreover, the chromosomal abnormality also became undetectable six months after the initiation of treatment.

The phagocytic index in the control mice was 20%, and treating the animals with danazol reduced this activity gradually as the doses increased. If an inspection of blood monocyte level is made, the first dose (200 µg\mouse) reduced the monocyte count and percentage (580 cells/cu.mm.blood and 8%, respectively), while the last three doses contributed to increased counts and percentages of these cells (an opposite picture of phagocytic index). So, danazol may interfere with the phagocytic activity, and to compensate such defect the blood monocyte count has to be increased. Recently, it has been found that a treatment of human peritoneal macrophages with danazol did not effect their phagocytic activity, but the author suggested that a high concentration of the drug may picture a decreased release of their cytotoxic substances (nitrates and TNF-alpha). The author supports its suggestion by the evident necrotic changes observed in the treated macrophages (10). A similar observation has been made by Magry and colleagues (11) to confirm this, although a different technical approach was employed. However, both observations shared some limitations, they were built in the ground of *in vitro* treatment. Therefore, a metabolic inactivation of danazol was not made, and its well-known fact that most drug activities are modulated positively or negatively after such inactivation in the liver. So the non-significant decrease in the Phagocytosis of the forthcoming observations may be explained in this context, and the present findings support such view, which was built on *in vivo* treatment. In

agreement with this, danazol has shown to reduce platelets phagocytosis in patients with idiopathic thrombocytosis (12, 13). A further support of the present findings has also been recently introduced, and adherence of erythrocytes to monocytes treated with danazol was reduced (14).

The spleen is an important secondary lymphoid organ, and represents a site of confrontation and response to blood-born pathogens. The mitotic activity of the splenic cells (lymphocytes) was also affected by the danazol treatment, and at a dose of 800 µg/mouse the mitotic index showed a reduced level of about 10% beyond the control value. However, lower doses (200, 400 and 600µg/mouse), the mitotic index showed a reduced level of about 10% beyond the control value. However, lower doses (200, 400 and 600 µg/mouse) showed some enhancement of such activity. No much information are available to confirm or/and explain this. But as the natural killer cells (NK) are being a type of lymphocytes, a recent investigation on rats with experimental endometriosis demonstrated a significant lower activity of splenic NK cells in these animals, and treating them with danazol recovered the activity to the level of intact rats (15).

In conclusion, the present data demonstrated *in vivo* that danazol might show some modulation (positively or negatively) of the immune system, functionally and structurally, and such effect is a dose-dependent. However, it is too early to generalize such conclusion and further investigations are required to include other and more advanced immunological parameters (i.e. cytokine levels and subsets of immune cells defined in terms of CD markers).

Table 1: Leucocyte counts (total and differential) in control mice and mice treated with danazol.

Animal Groups	Dose (µg/mouse)	Leucocyte Count/cu.mm.blood (mean±S.E.)				
		Total	Differential			
			Lymphocytes	Neutrophils	Monocytes	Eosinophils
Control	Zero	7525±229	3447±213 (51.1)	2668±69 (35.5)	693±19 (9.2)	245±37 (3.3)
Treated I	200	7250±202	3101±128* (42.8)	3389±97* (46.7)	580±29* (8.0)	200±19 (2.8)
Treated II	400	10625±427*	4057±134 (83.2)	4308±214* (40.5)	1892±126* (17.8)	370±53* (3.5)
Treated III	600	9300±216*	4445±312 (47.8)	3309±214* (35.6)	1790±57* (19.2)	44±14* (0.5)
Treated IV	800	6725±240*	3434±183 (51.1)	2119±86* (31.5)	1157±81* (17.2)	17±7* (0.3)

Note: Numbers in parentheses represent percentages of total means.

*: Significant difference compared to control.

Table 2: Phagocytic index (peritoneal cells) and mitotic index (bone marrow and spleen) in control mice and mice treated with danazol.

Animal Groups	Dose (µg/mouse)	Phagocytic Index (%; mean±S.E.)	Mitotic Index (%; mean±S.E.)	
			Bone Marrow	Spleen
Control	Zero	20.0±2.1	22.0±2.0	18.5±0.6
Treated I	200	18.1±0.6	21.5±0.6	20.3±1.1
Treated II	400	9.1±0.7*	31.5±0.7*	22.8±1.3*
Treated III	600	9.6±1.2*	27.3±1.1*	25.1±0.9*
Treated IV	800	7.3±0.3*	18.4±0.4*	16.7±0.7*

*: Significant difference compared to control.

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