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Endometrial Effects of Danazol in Perimenopausal Abnormal Bleeding

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INTRODUCTION

Danazol (D), a 2-3 isoxazol derivative of 17-ethinyltestosterone, is routinely used for the treatment of endometriosis[1] and is known to suppress menstrual bleeding.[2] Endometrial effects of D are not only related to the well-known inhibition of the hypothalamic pituitary function and ovarian steroid synthesis, but they seem to be primarily determined by a direct action on the endometrium.[3] In fact, D binds to progesterone receptors (PR) and inhibits 17-β-hydroxy-steroid-dehydrogenase[4] and steroid sulfatase activity[5] in human endometrium. The effectiveness of D in treatment of perimenopausal abnormal bleeding has been confirmed in many trials.[6-7] The demonstration of antiproliferative activity on human endometrial cancer cells *in vivo*[8] and on adenomatous hyperplasia *in vivo*,[9] together with the histologic observation that D induces rapid atrophic changes whereas a marked decidual reaction is found through progestin treatment, suggest a difference in the effect of D and typical progestins on endometrial tissue. In this regard we have considered useful to evaluate clinically, hysteroscopically and histopathologically the efficacy of D 200 mg daily for 3 months in the treatment of perimenopausal abnormal bleeding sustained by endometrial hyperplasia without cytologic atypia[10] in patients at their first therapeutic approach (group A) and in women already treated by progestin with persistance of the disease or recurrence within two months (group B). In addition a further endometrial antiproliferative mechanism of action of D, probably in relation with its immunosuppressive activity is indirectly shown by means of a primary endometrial cell culture conditioned by medium from peritoneal macrophages cultured *in vitro*.

MATERIALS AND METHODS

Subjects and Clinical Protocols

We have treated by 200 mg daily of D 42 patients with perimenopausal abnormal bleeding underlying an endometrial hyperplasia without cytologic atypia and not previously treated (group A) and 23 women with uterine bleeding already treated by Norethisterone and/or Medroxyprogesterone but with a persistance or recurrence of symptoms within two months (group B). Each patient underwent a hysteroscopic ex-

amination with guided endometrial biopsy before starting treatment. The treatment was monitored by: 1) a subjective semiquantitative evaluation of bleeding during treatment and in a follow up to 12 months; 2) hysteroscopically before, at the end of treatment, and in a follow up to 12 months; 3) histopathologically before and after treatment. Side effects and adverse reactions were carefully evaluated.

Experimental Section

On the basis of a previous experience[11] in which we showed that D, at a concentration of 10^{-6}M, significantly reduced the phagocytic ability of peritoneal macrophages *in vitro*, we wanted to see if the steroid is able to decrease the secretion of growth factors[12,13] by these immune cells and how such an immunosuppressive effect can be related to endometrial proliferation *in vitro*. Macrophages derived from peritoneal fluid collected laparoscopically with an average yield of 5×10^5 cells/ml. Cells were put in RPMI medium with 5% FBS steroid free, washed, centrifuged, and resuspended to a final concentration of 1×10^6 per dish using 24-well tissue culture plates in 1.5 ml of medium. Cultures were washed 2 hours after plating to separate nonadherent cells, and then incubated for 48 hours. Then supernatants were recovered and frozen at −80°C or immediately used. In some of these cultures, after separating nonadherent cells, D was added at different concentrations washing and changing medium 24 hours later. Then macrophages were cultured for 48 hours when supernatants were recovered. Endometrial cells derived from biopsy specimens sampled in the luteal phase and immediately put in ice-cold 1:1 mixture of DMEM and Ham's-F-12. Samples were dissected and a separation between stromal and epithelial cells was performed following the Osteen technique[14] with some slight modifications.[15] Essentially the method was based on three consecutive incubations in medium containing 0.5% collagenase and 0.05% DNAase followed by differential sedimentation of epithelial cells at unit gravity and by selective attachment of stromal cells to plastic cultureware. At the end of separation both epithelial and stromal cells were plated at a final density of 5×10^5 cells/ml in 24-well plates in DMEM-F-12 with 10% charcoal extracted FBS for 48 hours (T_0). Cultures were washed and incubated in medium with 2% steroid free FBS for 48 hours (T_f). Cell counts were performed on day 2 and day 4 of culture. At T_0 different amounts (10–60% vol/vol) of supernatant from macrophage culture previously treated or not with different concentrations of D were added to the endometrial cell cultures. Viability of cell cultures were assessed by Trypan blue exclusion.

RESULTS AND DISCUSSION

In group A (TABLE 1) there was a subjective significant decrease of bleeding in 38 out of 41 patients (88.2%), and this result remained unchanged (84.3%) 2 months after discontinuation of treatment, while 4 months after (24.9% of recurrence) and up to 12 months the abnormal bleeding reappeared in 56.2% of women. In TABLE 2 is shown how in group B (patients not responding to progestins) the persistence of heavy blood loss at the end of treatment was 22.7%; this can be considered a very good result considering patients as a control for themselves. This result was practically unchanged 2 months later (27%), with a significant increase in recurrence at 4 (49.9%)

TABLE 1. Blood Loss and Days of Bleeding in Group A

	Pretreatment n (%)	End of Treatment n (%)	Months Posttreatment 2 n (%)	4 n (%)	Up to 12 n (%)
Blood Loss					
Flooding	19 (45)	–	1 (3)	2 (7)	4 (25)
Heavy	23 (55)	3 (7)	3 (9)	6 (18)	6 (31)
Normal	–	33 (81)	25 (79)	18 (69)	6 (38)
Spotting	–	2 (5)	1 (3)	1 (3)	1 (8)
Amenorrhea	–	3 (7)	2 (8)	1 (3)	–
Number of Patients	42	41	32	28	16
Days of Bleeding (mean ± SD)	8.9 ± 3.7	5.2 ± 3.4	5.4 ± 3.8	6.3 ± 4.8	6.9 ± 3.7

and up to 12 months (55.3%). Hysteroscopically in only two patients in group A (4.8%) and two patients in group B (9%) did the hyperplastic picture not revert at the end of treatment, and one of these four patients was a false positive on the basis of the biopsy result (TABLE 3). The hysteroscopic follow-up showed a rapid appearance of hypoatrophic pictures with a progressive increase in the normal findings both proliferative and secretive, but also with a meaningful reappearance of hyperplasia up to 60% at 4 and 12 months (TABLE 4).

The comparison of hysteroscopic findings with histopathologic pictures shows clearly a good correlation with just 1 hysteroscopic false positive, 2 false negative, and 2 not diagnostic exams (TABLE 5). The most interesting aspect of this comparison is the invaluable importance of the hysteroscopy in the diagnosis of hypoatrophia, which is very often undiagnosed bioptically for inadequate sampling.

The most frequent side effects during this treatment were weight gain, muscle cramps, headache and acne, but in only two cases was it necessary to stop treatment (TABLE 6).

Our data clearly show the efficacy of the regimen of D 200mg daily in the treatment of perimenopausal abnormal bleeding, as indicated in previous reports too,[6,7,9] but

TABLE 2. Blood Loss and Days of Bleeding in Group B

	Pretreatment n (%)	End of Treatment n (%)	Months Posttreatment 2 n (%)	4 n (%)	Up to 12 n (%)
Blood Loss					
Flooding	8 (38)	1 (5)	2 (9)	3 (17)	3 (26)
Heavy	14 (81)	4 (18)	4 (18)	6 (33)	8 (53)
Normal	–	14 (63)	14 (83)	8 (44)	3 (28)
Spotting	–	1 (5)	1 (5)	–	–
Amenorrhea	–	2 (9)	1 (5)	1 (6)	1 (7)
Number of Patients	23	22	22	18	15
Days of Bleeding (mean ± SD)	8.5 ± 4.1	5.9 ± 2.2	6.4 ± 3.6	6.8 ± 4.3	7.6 ± 3.8

TABLE 3. Hysteroscopic Findings before (T_0) and at the End of Treatment (T_1) with Danazol 200 mg Daily for 3 Months

Hysteroscopic Finding	T_0		T_1	
	Group A n (%)	Group B n (%)	Group A n (%)	Group B n (%)
Hyperplasia	39 (92.8)	22 (95.6)	2 (4.8)	2 (9.0)
Hypo-atrophy	–	–	32 (78.0)	16 (72.7)
Normotrophy	2 (4.7)	–	6 (14.6)	4 (18.1)
Not diagnostic	1 (2.3)	1 (4.3)	1 (2.4)	–
TOTAL	42	23	41	22

TABLE 4. Hysteroscopic Follow-up at 2 Months (T_2), 4 Months (T_3) and up to 12 Months (T_4) after Treatment with Danazol 200 mg Daily for 3 Months

Hysteroscopic Finding	T_2		T_3		T_4	
	Group A n (%)	Group B n (%)	Group A n (%)	Group B n (%)	Group A n (%)	Group B n (%)
Hyperplasia	2 (11.1)	3 (20.0)	4 (28.5)	3 (27.2)	9 (60.0)	5 (62.5)
Hypo-atrophy	10 (55.5)	8 (53.3)	4 (28.5)	2 (18.1)	2 (13.3)	1 (12.5)
Normotrophy						
Proliferative	4 (22.2)	3 (50.0)	4 (28.5)	4 (36.3)	3 (20.0)	2 (25.0)
Secretory	2 (11.1)	1 (6.6)	2 (14.2)	2 (18.1)	5 (33.3)	–
Not diagnostic	–	–	–	–	–	–
TOTAL	18	15	14	11	15	8

TABLE 5. Comparison between Hysteroscopic Findings and Histopathologic Pictures before (T_0) and after Treatment (T_1)

Histopathologic Appearance	Hysteroscopic Finding									
	Hyper-plasia		Hermo-trophy		Hypo-atrophy		Not diagnostic		TOTAL	
	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1	T_0	T_1
Adenomatous hyperplasia	9	2	–	–	–	–	–	–	9	2
Glandular hyperplasia	21	1	2	–	–	–	2	–	25	1
Gland. cystic hyperplasia	31	–	–	–	–	–	–	–	31	–
Proliferative (a) with fibrotio stroma (b)	–	1a	–	7b	–	–	–	–	–	8
Irregular maturation	–	–	–	2	–	–	–	1	–	3
Atrophic	–	–	–	–	–	18	–	–	–	18
Atrophic with stroma pseudodecidualization	–	–	–	1	–	11	–	–	–	12
Inadequate sampling	–	–	–	–	–	19	–	–	–	19
TOTAL	61	4	2	10	–	48	2	1	65	63

TABLE 6. Side Effects during Treatment

	Month 1 n (%)	Month 3 n (%)
Weight gain	7 (10.7)	5 (7.9)
Headache	3 (4.6)	3 (4.7)
Nausea or vomiting	3 (4.6)	–
Acne	3 (4.6)	4 (6.3)
Hirsutism	1 (1.5)	1 (1.5)
Muscle cramps	4 (6.1)	2 (3.1)
Hot flushes	3 (4.6)	3 (4.7)
Skin rash	1 (1.5)	–
Voice change	1 (1.5)	1 (1.5)
Edema	2 (3.0)	–
Decreased breast size	3 (4.6)	4 (6.3)
TOTAL	65	63

they also point out the effectiveness of this steroid in those forms not responding to progestins, confirming indirectly the existence of additional mechanisms of antiproliferative action on the endometrium if compared to progestogens. In order to verify the hypothesis of an antiproliferative effect mediated by the immunosuppressive activity of this steroid, we have used an experimental model of primary endometrial cell cultures conditioned by supernatants from peritoneal macrophages cultured *in vitro* with or without D. The rationale underlying this hypothesis was to test the ability of D to inhibit the secretion of growth factors stimulating the endometrial proliferation by uterine immune cells.

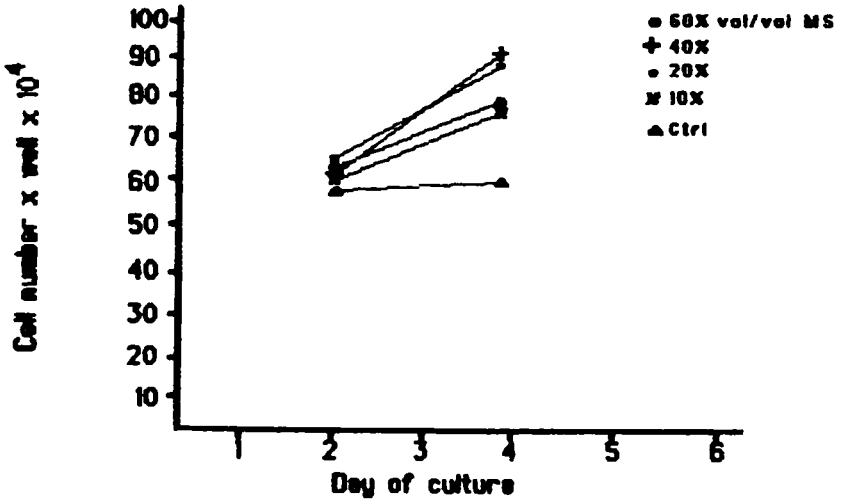

FIGURE 1. Epithelial cells growth with the addiction of different concentration of supernatant from a peritoneal macrophage culture (MS).

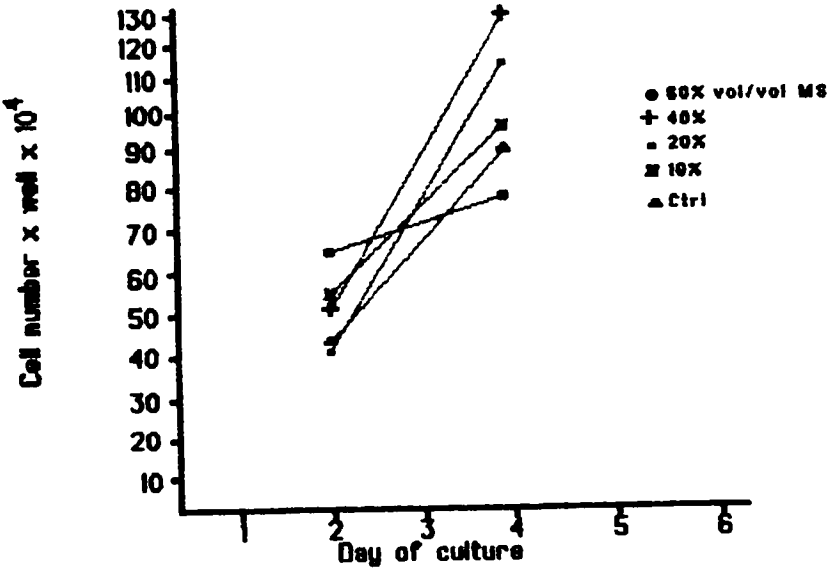

FIGURE 2. Stromal cells growth with the addiction of different concentration of supernatant from a peritoneal macrophage culture (MS).

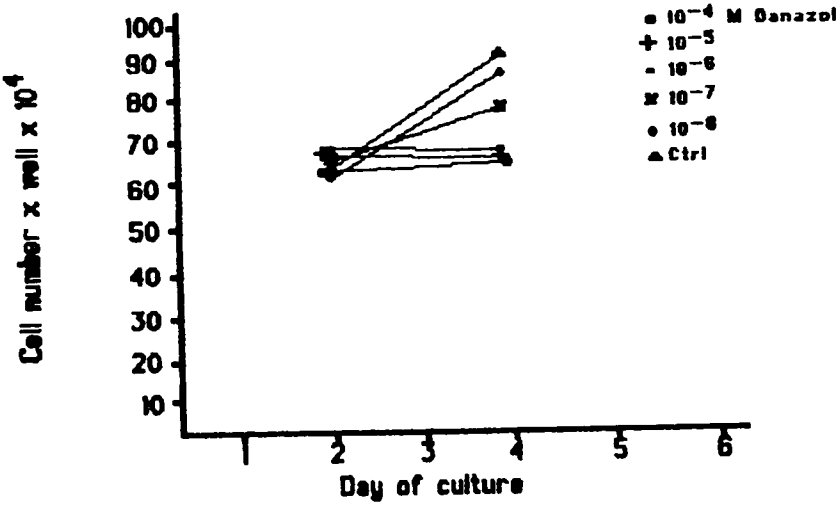

FIGURE 3. Epithelial cells with the addiction of 40% vol/vol of supernatant from a peritoneal macrophage culture previously treated with different concentration of Danazol.

Our results with this model have shown a marked increase in proliferation when adding to endometrial epithelial cells an aliquot of 40% in volume of supernant from the peritoneal macrophage culture (FIG. 1). We have used peritoneal samples as a source of macrophages because they can be easily collected and because they seem to be strictly related to the resident immune cells in human endometrium.[15] Using endometrial stromal cells the increase induced by macrophage supernatant was not so evident, probably in relation to the more active early proliferation to confluence of these cells *in vitro* (FIG. 2). When we have used supernatants of macrophages previously exposed to different concentrations of D, we have demonstrated a significant decrease in the proliferation with the peak at 10^{-6}M concentration of D (FIG. 3).

In conclusion, our clinical data strongly suggest the presence of other mechanisms of endometrial antiproliferative effect of D in addition to the progestin-like ones. Among these further mechanisms, our preliminary results using a conditioned endometrial primary cell culture seem to show the importance of the inhibition in the production of growth factors by local immune cells exerted by D.

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