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โครงการ

การศึกษาผลของยาดอกซี่ซัยคลินในการรักษาภาวะเลือดออกผิดปกติจากโพรงมดลูก ในสตรีที่ใช้ยาฉีดคุมกำเนิดโปรเมดรอกซี่โปรเจสเตอโรนอะซีเตท

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A randomized, double-blind, placebo-controlled trial of doxycycline in depot medroxyprogesterone acetate-induced endometrial breakthrough bleeding.

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Running title: Doxycycline and bleeding in DMPA users.

Abstract

BACKGROUND: Unpredictable endometrial bleeding disturbances due to depot-medroxyprogesterone acetate (DMPA) may be caused by endometrial matrix metalloproteinase (MMP) release and decreased tissue inhibitors (TIMP). Doxycycline is a potent inhibitor of MMP. This study aimed to study the effect of doxycycline on DMPA induced endometrial breakthrough bleeding. METHODS: Two hundred and fifty DMPA users who had frequent or prolonged endometrial bleeding were randomized into 5 groups. Group A was taken 50 mg. of doxycycline per day for 28 days. Group B was taken 200 mg. of doxycycline per day for 28 days. Group C was taken 50 mg. of doxycycline per day for 7 days and placebo for 21 days. Group D was taken 200 mg. of doxycycline per day for 7 days and placebo for 21 days. Group E was taken only placebo for 28 days. Numbers of bleeding days before and after treatment were compared. Endometrial biopsies were collected before and after treatment for immunohistochemical study of MMP-9, TIMP-1, TIMP-2 and TIMP-3 expression. RESULTS: 250 DMPA users were recruited, 5 DMPA users were loss follow up and 133 DMPA users had adequate endometrial tissue for immunohistochemical study. The number of bleeding days during 28 days of treatment and 30 days after treatment were

significantly decreased after treatment. The endometrial MMP-9 expression was significantly decreased and TIMP-3 expression was significantly increased after treatment. CONCLUSIONS: Doxycycline decreased the numbers of bleeding days, decreased MMP-9 expression and increased TIMP-3 expression in bleeding DMPA users. Further study should be required to study longer duration, more than 28 days, of doxycycline for treatment of endometrial bleeding in DMPA users.

Keywords: depot medroxyprogesterone acetate/ DMPA/ endometrial bleeding/ matrix metalloproteinase/ doxycycline.

Introduction

Depot-medroxyprogesterone acetate (DMPA) is the most popular long-acting progestogen-only contraceptive. Thirteen million women worldwide currently use DMPA for contraception (d'Arcangues,2000). Injection of 150 mg of DMPA every 84-90 days provides extremely high contraceptive efficacy. Failure rates range from 0 to 0.7 per 100 woman-years, which is comparable with surgical sterilization (Kaunitz,2001). However, bleeding disturbances from DMPA and the other progestogen-only contraceptives are the main reasons for discontinuation. The discontinuation rate of DMPA due to bleeding disturbances was approximately 21-28 per 100 women at one year of use, accounting for 52-68% of all reasons for discontinuation (Fraser,1999). Many treatments have been tried with unsuccessful results because the mechanisms of bleeding disturbances related to progestogen-only contraceptives are still not well understood (d'Arcangues,2000, Hickey and Fraser,2000).

Matrix metalloproteinases (MMP) are a family of zinc-dependent proteases which degrade extracellular matrix components (Curry and Osteen,2001, Henriet*, et al.*,2002). Twenty-six MMPs have been discovered. Regulation of MMPs is complex and occurs at multiple levels including gene transcription, a cascade of activation in which

proteases, including some MMPs, are able to activate the latent MMPs and inhibition by tissue inhibitors of metalloproteinases (TIMP). TIMPs bind active MMPs and form 1:1 complexes. Four TIMPs have been discovered. Changes in the balance between endometrial MMPs and TIMPs, in favour of greater MMP action, results in endometrial breakdown (Marbaix*, et al.*,1995, Marbaix*, et al.*,1996, Salamonsen and Woolley,1996, Zhang and Salamonsen,2002). Endometrial MMP expression varies during the menstrual cycle, with a marked increase in some MMPs observed perimenstrually. In an *in vitro* model, inhibition of MMP prevented breakdown of endometrial explants (Marbaix*, et al.*,1996). MMPs are postulated to be involved in the endometrial breakdown observed at menstruation (Marbaix*, et al.*,1995, Marbaix*, et al.*,1996, Salamonsen and Woolley,1996, Zhang and Salamonsen,2002).

Previous studies have shown altered MMP and TIMP expression and activation in endometrial biopsies from women using Norplant (Galant*, et al.*,2000, Marbaix*, et al.*,2000, Vincent*, et al.*,1999, Vincent*, et al.*,2000). One study has explored MMP and TIMP expression in DMPA users. This study found that women using DMPA had similar number of MMP-9 positive cells and decreased endometrial TIMP-1, TIMP-2 and TIMP-3 expression compared to normal perimenstrual controls. (Vincent*, et al.*,2002).

Doxycycline is a potent MMP inhibitor. Doxycycline has been used to treat the diseases which have over expression of MMP such as arthritis, periodontal diseases, aortic aneurysm and prevention of metastasis in some malignant diseases.

In this study, we aimed to study the effect of doxycycline on number of bleeding days and endometrial MMP expression in DMPA users who had frequent or prolonged endometrial breakthrough bleeding.

Materials and methods

Subjects

Two hundred and fifty healthy, fertile women, aged between 18 and 40 years, using DMPA for 12 to 24 weeks, who had frequent or prolonged endometrial bleeding were recruited from the Family Planning Clinic at King Chulalongkorn Memorial hospital between July 2003 and January 2008. Frequent or prolonged endometrial bleeding defined as bleeding episodes which were more frequently than 4 times or lasted longer than 10 days in the last 90 days (Belsey and Pinol,1997). All subjects had no known medical disorders, gynaecological disorders, previous history of chronic anovulation or allergy to doxycycline and tetracycline. Subjects reporting any other hormonal treatment

were excluded. All subjects were screened by transvaginal ultrasonography and subjects who had abnormal endometrial sonogram, e.g. endometrial polyp or submucous myoma, were excluded. All subjects had received 150 mg. of DMPA for contraception, with repeated injection approximately every 84 days. Menstrual data were prospectively recorded on a daily menstrual diary chart. Subjects who had missed more than 10 days of menstrual data were excluded. Ethical approval for the study was obtained from the Ethics Committee of the Chulalongkorn University.

Subjects were randomized into 5 groups. Group A was taken 50 mg. of doxycycline per day for 28 days. Group B was taken 200 mg. of doxycycline per day for 28 days. Group C was taken 50 mg. of doxycycline per day for 7 days and placebo for 21 days. Group D was taken 200 mg. of doxycycline per day for 7 days and placebo for 21 days. Group E was taken only placebo for 28 days.

Numbers of bleeding days before and after treatment were compared. Endometrial specimens were obtained by Endocell biopsy (Wallach Surgical Devices, Inc., USA) before and after finished treatment. All endometrial tissue samples were fixed overnight in 10% buffered formalin and paraffin embedded using a routine method. Five

micron sections were collected on 3-aminopropyltriethoxysilane-coated slides for immunohistochemical study of MMP-9, TIMP-1, TIMP-2 and TIMP-3 expression.

Immunohistochemistry

The sections on 3-aminopropyltriethoxysilane-coated slides were deparaffinized and rehydrated to distilled water. Antigen retrieval was done for MMP-9, TIMP-1, TIMP-2, TIMP-3 immunostaining by heating the sections in Target Antigen Retrieval Solution® (Dako Corp., USA) in a microwave oven set on high power for 15 minutes. Slides were washed in 0.05 M Tris-buffered saline (TBS) and endogenous peroxidase was blocked with 3% hydrogen peroxide. Nonspecific binding of the primary antibody was blocked by incubating the slides with 5% swine serum for 5 minutes.

Primary antibody for MMP-9 (diluted 1:40, NCL-MMP9, Clone 2C3, Novocastra Laboratories) were applied to the sections and incubated at room temperature for 60 minutes. Primary antibody for TIMP-1 (diluted 1:1,000, AB8228, Chemicon, USA), TIMP-2 (diluted 1:100, MAB13446, Clone 3A4, Chemicon) and TIMP-3 (diluted 1:100, AB19122, Chemicon) were applied to the sections and incubated at room temperature for 30 minutes.

Biotinylated anti-mouse/rabbit antibody and streptavidin conjugated to horseradish peroxidase (DAKO LSAB® + Kit, Dako Corp.) were then added sequentially and incubated for 15 minutes each. Staining was visualized by addition of substrate and chromogen 3,3'-diaminobenzidine (DAKO® Liquid DAB+, Dako Corp.) for 5 minutes. All tissue sections were counterstained with Mayer's haematoxylin, dehydrated and cleared in xylol then mounted with Ultramount.

Menstrual phase endometrium sections were used as positive controls for MMP-9 and TIMP-3 immunostaining. Colonic adenocarcinoma sections were used as positive controls for TIMP-1 and TIMP-2 immunostaining. For negative controls, the sections were incubated with IgG from same animal species which produced primary antibodies (mouse or rabbit IgG) at a matched concentration in place of primary antibody.

Assessment of immunostaining

Assessment of immunostaining was performed as described previously (Sereepapong*, et al.*,2004). All tissue sections were blindly assessed by the same observer (W.S.).

Positive immunostaining of MMP-1, MMP-9, TIMP-1, TIMP-2 and TIMP-3 was located in the cytoplasm of cells. Therefore, it was often difficult to count the individual

positive-stained cells. Thus we used the modified H-score for assessment (Ravn*, et al.*,1993). Firstly, the fraction (F) of stained cells in each compartment was estimated: 0 = 0-9%, 1= 10-39%, 2 = 40-69%, 3 = 70-89%, and 4 = 90-100%. Secondly, the staining intensity (I) was scored: 0 = no staining, 1 = weak but definite staining, 2 = moderate staining, 3 = pronounced staining and 4 = intense staining. Finally, the modified H-score was calculated by the formula $\sum$(FxI)/4. Endometrial glands and stroma were assessed separately. We have validated the counting and modified H-score techniques as shown by high intraobserver and interobserver correlation in our previous studies (Sereepapong*, et al.*,2004).

Statistical analysis

Data were analyzed using the Statistic Package for Social Science (SPSS) software for Windows, version 11.5 (SPSS Inc., USA). Comparisons between groups were made using the mean, standard deviation and one way ANOVA for normal distribution variables. Median and Kruskal-Wallis H were used for non-parametric variables. *P* values of less than 0.05 were considered to indicate statistically significant differences.

Results

Two hundred and fifty DMPA users were recruited. Five subjects were loss follow up, two in group A, one in group C and two in group D. The age, body mass index and numbers of bleeding days were not significantly different between groups as shown in Table 1.

The numbers of bleeding days during the 28 days of treatment period were significantly different between groups. The numbers of bleeding days during the 30 days post-treatment period were also significantly different between groups. The numbers of bleeding days during the 90 days post-treatment period were not significantly different between groups as shown in Table 2.

Only 133 subjects had adequate endometrial tissue for immunohistochemical study due to well known suppression of endometrial growth by DMPA. The MMP-9 expression was significantly decreased after treatment between groups. The TIMP-3 expression was significantly increased after treatment between groups. The TIMP-1 and TIMP-2 expression were not significantly different after treatment between groups as shown in Table 3.

Table 1 Data before treatment.

	Group A	Group B	Group C	Group D	Group E	*P* Value
Age (years)[a]	26.2±5.4	26.9±5.4	27.1±2.9	28.2±7.3	24.7±6.3	NS[c]
BMI (kg/m^2)[a]	21.8±2.2	23.1±3.6	21.7±2.9	22.0±2.5	21.5±2.5	NS[c]
No. of bleeding day before treatment[b]	28.5	28.5	30.0	28.0	32.0	NS[d]

[a]mean ± SD, [b]median, [c]one way ANOVA, [d]Kruskal-Wallis

Table 2 Number of bleeding day during and after treatment.

No. of bleeding day	Group A	Group B	Group C	Group D	Group E	*P* Value
during treatment[b]	9.0	11.0	6.0	5.0	4.0	<.05[d]
30 days after treatment[b]	14.0	11.0	12.0	6.0	5.0	<.05[d]
90 days after treatment[b]	15.0	14.0	12.5	12.0	13.5	NS[d]

[a]mean ± SD, [b]median, [c]one way ANOVA, [d]Kruskal-Wallis

Table 3 Immunohistochemical study after treatment.

H-Score	Group A	Group B	Group C	Group D	Group E	*P* Value[b]
No. of cases	48	50	49	48	50	-
No. of adequate tissue	24	25	28	29	27	-
MMP9 H-score[a]	3.0	1.5	3.0	1.5	0	<.001
TIMP1 H-score[a]	1.5	6.0	6.0	4.0	4.0	NS
TIMP 2 H-score[a]	6.0	4.0	4.0	2.0	2.0	NS
TIMP 3 H-score[a]	0.5	3.5	3.0	2.0	3.5	<.05

[a]median, [b]Kruskal-Wallis

Discussion

This study has demonstrated that the numbers of bleeding days during 28 days treatment period and 30 days post-treatment period were significantly decreased, especially in group D which received doxycycline 50 mg. for 28 days and group E which received doxycycline 200 mg. for 28 days. In group A (placebo), group B (doxycycline 50 mg for 7 days) and group C (doxycycline 200 mg. for 7 days) had less effect.

The immunohistochemical study of MMP-9 and TIMP-3 also showed changing of expression after treatment in favor of less tissue breakdown in group B, C, D and E compared to group A which is placebo group. This result confirmed the finding of decreased number of bleeding day after treatment in group D which received doxycycline 50 mg. for 28 days and group E which received doxycycline 200 mg. for 28 days. This effect in group B and C may be not strong enough to have clinically less bleeding compared to placebo group.

We recommend using doxycycline 50 mg. or 200 mg per day for 28 days as an alternative treatment in frequent or prolonged endometrial breakthrough bleeding in DMPA users.

Belsy *et al.* have reported that bleeding patterns in DMPA users varied with ethnic groups (Belsey and Peregoudov,1988). In this study, we recruited only Thai DMPA users. There may be some limitations of generalising our results to other ethnic groups.

In conclusion, doxycycline 50 mg. and 200 mg for 28 days can decreased the number of bleeding day in DMPA users who have prolonged or frequent endometrial breakthrough bleeding. This effect was confirmed by the immunohistochemical study of endometrial MMP and TIMP expression.

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