A comparative study of Cycloferm® and depot medroxyprogesterone acetate (DMPA) effects on endometrial vasculature

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Abstract

Objectives The most common reason for discontinuation of long-acting progestogen-only contraceptives is irregular bleeding following local endometrial vascular changes. To reduce unpredictable bleeding episodes among depot medroxyprogesterone acetate (DMPA) users, the combined injectable contraceptive, Cycloferm®, was offered as an alternative. However, there is a gap in our knowledge about the effects of Cycloferm on the endometrial vasculature and patterns of bleeding. This study aimed to compare the effects of Cycloferm and DMPA on endometrial vascular density, endometrial histology and pattern of bleeding.

Methods Sixty-eight healthy women with regular menstrual bleeding and seeking injectable long-acting contraceptives were recruited. Two endometrial samples (before and 3 to 6 months after initial exposure to DMPA or Cycloferm) were collected from each participant. The samples were stained using an immunohistochemical method and anti-CD34 to visualise the endometrial vasculature. Endometrial vascular density was assessed using standard techniques.

Results Sixty-eight women were randomly assigned to Cycloferm (38 women) or DMPA (30 women). Endometrial vascular density was 149.3 ± 6.7 (mean $\pm$ SD)/mm² before

injection. This significantly decreased to 132.4 ± 12.2 after DMPA use, and from 151.9 ± 5.8 to 131.8 ± 12.8 vessels/mm² following Cycloferm use (paired *t*-test, *p* < 0.05). However, there was no significant difference between endometrial vascular density during treatment with Cycloferm or DMPA. Total bleeding days in the first and second 3-month time intervals were 28 ± 23 and 18 ± 12 days in DMPA users and 22 ± 14 and 16 ± 9 days in Cycloferm users, respectively. Spotting was the most common type of bleeding experienced, and atrophic endometrium was the most common histological pattern observed in both groups.

Conclusions This study demonstrated that both Cycloferm and DMPA use are associated with decreased endometrial vascular density and atrophic endometrium, in addition to irregular bleeding, mainly spotting. There was no significant difference in bleeding patterns or endometrial findings observed for these two injectable contraceptives in Iranian women.

Keywords Cycloferm®, DMPA, endometrial vasculature, injectable contraceptive methods, irregular bleeding

J Fam Plann Reprod Health Care 2007; **33**(4): 271–276
(Accepted 13 August 2007)

Introduction

Progestogen-only contraceptives provide a safe and highly effective method of fertility regulation. Unfortunately, they are commonly associated with the problem of endometrial breakthrough bleeding, often leading to discontinuation of use.¹ Depot medroxyprogesterone acetate (DMPA) is an aqueous suspension of pregn-4-ene-3,20-dione,17-(acetyloxy)-6-methyl-(6 α) administered by intramuscular injection (150 mg once every 3 months) for long-term contraception. MPA is detected in the serum within 30 minutes following injection. Serum concentrations generally plateau at about 1.0 ng/ml for about 3 months, after which there is a gradual decline.² Totally unpredictable changes in menstrual pattern are the most frequently indicated reasons for discontinuing DMPA use.³ So, the main reason for the development of combined once-a-month injectable contraceptives was the need for a

Key message points

- Depot medroxyprogesterone acetate (DMPA) and Cycloferm® cause marked and similar atrophic endometrial changes, with significantly decreased endometrial vascular density and formation of dilated, thin-walled, superficial vessels (which may contribute to the mechanism of abnormal bleeding).
- The irregular uterine bleeding and spotting patterns were very similar with both preparations.
- It appears that the oestrogen component of Cycloferm has little effect on the short- to medium-term endometrial appearances or bleeding patterns compared with DMPA alone.

long-acting injectable method that produces a more regular vaginal bleeding pattern. The extensive literature indicates that although combined oestrogen-progestogen once-a-month injectable contraceptives such as Cycloferm® (25 mg MPA and 5 mg oestradiol cypionate) usually produce more regular bleeding patterns than long-acting progestogen-only injectable contraceptives such as DMPA, the patterns are by no means normal.⁴ Although menstrual disturbances occur less often in Cycloferm users versus DMPA users,⁴ they are still the leading medical reason for discontinuation.^{5,6} Once-a-month injectable contraceptives including Cycloferm are generally well accepted, even though women must attend a clinic every 27–33 days for an injection.⁷

Disturbances of menstrual bleeding in users of long-acting progestogen-only contraceptives are associated with local changes in the endometrial microvasculature. The mechanism of this bleeding is unknown, and it is not directly related to changes in circulating levels of endogenous or exogenous steroid hormones.⁸ Changes in

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the endometrial vasculature have been identified after prolonged exposure to progestogens alone,⁹⁻¹¹ but have not been studied following monthly combined injectable methods.

An increase in endometrial microvascular density has been observed after Norplant® (low-dose levonorgestrel subdermal implant) exposure,^{11,12} while a decrease has been demonstrated in women exposed to high- and medium-dose progestogens.¹³ In women treated with the very different hormonal system, danazol, or a gonadotropin-releasing hormone agonist, or after menopause, vascular density in the consequent atrophic endometrium appears to be constant.¹⁴ It seems that exogenous sex steroids may disrupt the normal, tightly controlled relationship between the growth of endothelial cells and capillaries and that of glandular and cellular components of the endometrium.^{12,14}

This study aimed to compare endometrial microvascular density during the early months of DMPA and Cyclofem exposure, and determine whether there were any objective endometrial vascular changes to compare with adverse bleeding patterns.

Methods

In an experimental study, 68 women aged between 18 and 39 years, who had regular menstrual cycles and who required long-term contraception, were recruited between July 2001 and September 2003 in a family planning centre at Qazvin Medical Science University, Tehran, Iran. Subjects were randomly assigned to use either Cyclofem (38 women) or DMPA (30 women). Eight extra subjects were added to the Cyclofem group on the basis that because this was a newly introduced monthly contraceptive, a greater number of dropouts were predicted to occur in this group. The investigators put 68 marbles in a bag and the women randomly selected one of 38 red marbles (Cyclofem group) or 30 yellow marbles (DMPA group). Then the subjects were fully counselled and were specifically informed of the likely occurrence of bleeding irregularities. Written informed consent was obtained from participants before any investigations commenced. Participants had the option to leave the study at any time.

DMPA and Cyclofem

DMPA (150 mg depot medroxyprogesterone acetate; Pfizer, New York) and Cyclofem (25 mg MPA and 5 mg estradiol cypionate; Farmaceuticas, Mexico) were started during the first 7 days after menstrual bleeding, and injected every 3 months for DMPA and once a month for Cyclofem users, using a 2 or 5 ml syringe and a 21- to 23-gauge intramuscular needle into the deltoid muscle of participants.

Menstrual bleeding patterns

Subjects prospectively recorded "bleeding" or "spotting" on a menstrual chart daily. Bleeding was defined as "any bloody vaginal discharge that requires the use of such protection as pads and tampons" and spotting as "any bloody vaginal discharge that is not generally great enough to require sanitary protection".¹⁵

Endometrial histology and immunohistochemistry

One endometrial biopsy was taken during the secretory phase of the pre-treatment (control) cycle. A second biopsy was planned to be taken from each subject between 3 and 6 months after starting DMPA or Cyclofem. The biopsies in Cyclofem users were taken as close to 2 weeks after the previous injection as possible to coincide with the end of a sustained period of oestrogen exposure. The biopsies were

taken using the Pipelle® Suction Curette (Prodimed, Cornier, Neuilly-en-Thelle, France).

Endometrial histological appearance

Biopsies were immediately placed in 10% buffered formalin at 4°C for 4-6 hours, and then rinsed and stored in phosphate-buffered saline (PBS) at 4°C until processing. Endometrial tissues were processed by paraffin embedding, and 3 µm sections were cut onto silanised slides for either immunohistochemistry or haematoxylin and eosin (H&E) staining. One experienced pathologist performed all the histopathological evaluations. Control biopsies were classified according to the criteria of Noyes *et al.*¹⁶ For experimental biopsies, the following histological classifications were used:¹² (1) *proliferative* – features mainly consistent with the proliferative phase of the normal menstrual cycle; (2) *secretory* – features mainly consistent with the secretory phase of the normal cycle; (3) *shedding* – major evidence of shedding, including tissue breakdown and fibrin thrombi but with no evidence of a previous secretory phase; (4) *atrophic* – atrophic endometrium with very little dense stroma, reduced glands with small, cuboidal epithelial cells; and (5) *progestogenic* – evidence of exogenous progestogenic effects, small glands with cuboidal or low columnar epithelium, and pseudodecidualised stroma.

Immunohistochemistry

The Dako EnVision® polymer two-step detection system (K1393; Dako, Carpinteria, CA) was used for immunohistochemical staining of endometrial vascular structures. The visualisation system uses a pre-diluted peroxidase-labelled polymer conjugated with secondary antibodies to rabbit and mouse immunoglobulins. The endometrial microvasculature was visualised using the mouse monoclonal antibody against human CD34 antigen (Q Bend 10; Novacastra, Newcastle, UK), which is expressed on the endothelial cell membrane. After dewaxing and rehydration, the tissue sections were quenched for endogenous peroxidase activity (0.03% hydrogen peroxide) for 5 minutes at room temperature. After a buffer rinse, an innocuous protein solution (5% normal goat serum) was applied for 5 minutes at room temperature to block non-specific staining of the charged sites in collagen and connective tissue. Tissue sections were incubated with the CD34 antibody for 30 minutes at room temperature. The substrate chromagen was AEC-Red (Universal EnVision® System, Peroxidase AEC version K1393; Dako, Carpinteria, CA), applied for 10 minutes at room temperature. A negative control of non-immune mouse serum substituted for the primary antibody and a positive control of normal endometrium were used in each staining run.

All sections were counterstained with Mayer's haematoxylin (Merck, Darmstadt, Germany), mounted with an aqueous mounting medium (Paramount®; Dako, Carpinteria, CA) and examined by light microscopy with a grid eyepiece (Olympus BH2; Olympus Optical, Tokyo, Japan). All staining processes were performed in the Tehran Institute of Cancer.

Vessel counting

Samples were considered suitable for counting if the H&E-stained sections contained at least 10 random unit areas that could be counted at a magnification of ×400. All red-coloured structures were considered positive, even if a lumen could not be identified. All of the slides were blindly counted using the same microscope and using a grid eyepiece pre-calibrated with a slide micrometer. Two

Figure 1 CONSORT statement flow diagram detailing subject numbers in each phase of the study. DMPA, depot medroxyprogesterone acetate

individuals blindly counted the slides using a consistent technique. The Pearson correlation coefficient between the two researcher's counts was 93% ($p < 0.05$).

Ten random fields of view were assessed and the number of CD34-positive vessels was counted under high-power magnification ($\times 400$) using an eyepiece micrometer. Each field of view using the eyepiece micrometer corresponded to $25 \mu\text{m}^2$. A mean for each section was obtained using the 10 observations. This was used in the final calculation of the number of vessels per square millimetre. Photographs were taken with an Olympus digital camera model C2 500L. Vessel counting and photography were performed in the Tehran Ave Sina Fertility and Infertility Research Center.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL). Paired t -test was used to compare endometrial vascular density before and after injection of the contraceptives. Student's t -test was also used to compare endometrial vascular density and also bleeding days between Cyclofem and DMPA users. Analysis of variance (ANOVA) and the Tukey-Kramer post-hoc test were used to compare endometrial vascular densities in different histological categories or different patterns of bleeding. Pearson correlation was used to assess the correlation between vascular density and

spotting/bleeding days. The confidence interval for all the tests was 95%.

Ethical approval

The Ethics Committee of the Ministry of Health and the authorities of the National Medical Research Center approved the study.

Results

The average age of participants was 26 ± 3 (mean $\pm$ SD) years (range, 18–39 years). The groups did not differ as regards their mean age, as well as their data for parity, gravity and abortion (Table 1). Using Student's t -test and with a 95% confidence interval (CI), there was no evidence of any difference between the two groups ($p < 0.05$). All the participants reported a negative cervical smear in the last 2 years and bimanual pelvic examination was normal. No pregnancies occurred during the study period.

Endometrial histology

Sixty-two control endometrial biopsies, of acceptable size and quality, were taken from a total of 68 participants between July 2001 and September 2003. The CONSORT flow diagram (Figure 1) describes the exclusion of inadequate biopsy samples and loss to follow-up of subjects. Table 2 shows the endometrial histological appearance of the contraceptive users before and after contraceptive use.

Table 1 Demographic and obstetric characteristics of the injectable contraceptive (Cyclofem® and DMPA) users

Characteristic	Contraceptive			
	Cyclofem		DMPA	
	Mean	SD	Mean	SD
Age (years)	25.7	5.7	26.8	5.2
Age at marriage (years)	18.2	4.0	17.7	2.8
Age at first pregnancy (years)	19.1	3.5	18.1	4.3
Gravidity (<i>n</i>)	1.9	1.2	2.1	1.1
Parity (<i>n</i>)	1.8	1.0	1.9	0.8
Abortions (<i>n</i>)	0.2	0.4	0.2	0.4
Live children (<i>n</i>)	1.6	1.0	1.9	0.9
Age at menarche (years)	13.4	1.2	13.5	1.3
Duration of menses (days)	28.7	3.0	28.9	3.5
Weight (kg)	62.4	12.4	61.2	11.0
Systolic BP (mmHg)	108.4	20.4	106.3	10.4
Diastolic BP (mmHg)	60.6	7.0	59.1	9.0

BP, blood pressure; DMPA, depot medroxyprogesterone acetate; SD, standard deviation.

Twenty 'treatment' biopsies were obtained from Cyclofem users and 23 second biopsies from DMPA users. Several of these biopsies were very small and inadequate for meaningful immunohistochemical assessment.

Endometrial vascular density

The staining of the positive control sections was specific with no background staining, and all relevant negative controls showed no staining. Vessel counting was performed by two blinded researchers. Final counts were taken as the average of these two observers.

Paired *t*-tests demonstrated a significant decrease in endometrial vascular density from 151.9 ± 5.8 to 131.8 ± 12.8 (95% CI of difference 12.2–42.8, $p < 0.05$) after Cyclofem exposure, and also a significant decrease from 149.3 ± 6.7 to 132.4 ± 12.2 after exposure to DMPA (95% CI of difference CI 4.2–28.2, $p < 0.05$).

Student's *t*-test also demonstrated no significant difference between the endometrial microvascular density of DMPA and Cyclofem users during treatment (difference 0.6, 95% CI –5.8 to 8.4, $p = 0.88$).

Figure 2 shows an example of normal endometrial vascular density in the secretory phase before contraceptive use. Figures 3 and 4 demonstrate decreased endometrial vascular density after Cyclofem and DMPA use, respectively. These figures also demonstrate the presence of dilated, thin-walled superficial microvessels, which have been reported in other progestogen-only contraceptive users.

In the first 3 months of use, the mean $\pm$ SD days of

Figure 2 Normal secretory phase endometrium before contraceptive use (original magnification $\times 200$)

spotting, light, moderate and heavy bleeding were 15 ± 17.1 , 6.3 ± 8.3 , 4.0 ± 4.3 and 2.5 ± 6.7 in DMPA users, respectively, and were 12 ± 11.6 , 6.7 ± 6.2 , 2.6 ± 1.9 and 3.0 ± 6.6 in Cyclofem users, respectively. There were no significant differences between the patterns of bleeding, either during the first or second 3-month treatment periods with the two contraceptives (independent *t*-test, $p > 0.05$) (Figure 5). There was no correlation between vascular density and days of bleeding (Spearman correlation = 0.31, $p > 0.05$).

Discussion

For the first time, this study has compared endometrial microvascular changes during 3 to 6 months use of two different long-acting injectable contraceptives, DMPA

Figure 3 Decreased microvascular density after Cyclofem® use, with thin epithelium, small glands and a dilated, thin-walled vessel (arrow) (original magnification $\times 200$)**Table 2** Endometrial histological appearance of the injectable contraceptive users before and after contraceptive use

Endometrial histological appearance	Contraceptive [<i>n</i> (%)]		
	Cyclofem	DMPA	Total
Before use			
Proliferative	12 (32)	16 (53)	28 (41)
Secretory	22 (58)	12 (40)	34 (50)
Missed	4 (10)	2 (7)	6 (9)
Total	38 (100)	30 (100)	68 (100)
After use			
Atrophic	13 (34)	17 (57)	30 (44)
Proliferative	1 (3)	1 (3)	2 (3)
Progestogenic	0 (0)	2 (7)	2 (3)
Basal or myometrium	3 (8)	3 (10)	6 (9)
Missed	21 (55)	7 (23)	28 (41)
Total	38 (100)	30 (100)	68 (100)

Figure 4 Decreased microvascular density after depot medroxyprogesterone acetate (DMPA) use, with thin epithelium, small glands and a dilated, thin-walled vessel (arrow) (original magnification $\times 200$)

(progestogen-only) and Cyclofem (once-a-month combined oestrogen-progestogen), and has demonstrated a significant decrease in endometrial vascular density in both groups of users. However, there was no significant difference in the microvascular density of the two groups during exposure to these different injectable contraceptives.

Endometrial vascular density has been studied by several groups of investigators with a view to understanding the mechanisms of breakthrough bleeding in progestogen-only contraceptive users. A significant decrease in mean endometrial vascular density was observed in levonorgestrel intrauterine system users,¹⁷⁻²⁰ whereas an increase was observed after insertion of levonorgestrel subdermal implants (Norplant).^{11,12} Similarly, endometrial vascular density in mice increased significantly after treatment with subcutaneous Silastic® implants containing either MPA or levonorgestrel, compared with normal cycling mice.²⁰ It seems that changes in endometrial vascular density can be variously influenced by hormone type, dose and delivery system, with higher dosage systems being associated with decreased endometrial vascular density. It appears that the addition of 5 mg oestradiol cypionate to MPA in Cyclofem does not prevent or alter DMPA-induced endometrial microvasculature changes.

Endometrial dilated superficial microvessels, which have been hypothesised to be the site of breakthrough bleeding,¹⁴ were observed in several endometrial biopsies of DMPA and Cyclofem users. Dilated vessels, as well as endothelial cell columns without any lumen, and without basement membrane components, were demonstrated in a three-dimensional study of vessels in the endometrium of Norplant users.²¹ Similar vascular changes were also seen in the present study. Dilated vessels have been reported in the endometrium of users of long-acting progestogen-only contraceptives, such as Norplant, and the levonorgestrel-releasing intrauterine system in other studies.^{11,14,22,23} Although immunohistochemical studies like the present one have some limitations, such as limited and superficial biopsy sampling, they have been able to regularly show dilated vessels in these small endometrial samples.

Hysteroscopy has demonstrated dilated superficial endometrial vessels in one-third of Norplant cases. The presence of these dilated vessels was associated with more breakthrough bleeding in the previous 30 days.¹⁴ Dilated and fragile vessels appeared to be the probable site of the breakthrough bleeding.¹⁴ A decrease in the basement membrane components of endometrial vessels was also demonstrated.²² In Norplant users the endometrial endothelial basement membranes were deficient in collagen IV, laminin and heparan sulphate proteoglycans in the initial months of exposure, when bleeding problems are most common.²² Immunohistochemical studies on users of low-dose progestogen-only methods have demonstrated substantial changes in superficial endometrial vascular morphology, density and fragility. In addition, hysteroscopic studies have confirmed the increased fragility of these superficial vessels.²⁴

The roles of different endometrial molecular mechanisms, and cell types such as leukocytes, and the way in which they interact to produce troublesome breakthrough bleeding in long-acting hormonal contraceptive users are far from clear. There is evidence to implicate disturbed endometrial angiogenesis and the regulation of vascular supply to the tissue, exemplified by the variations in microvessel density, and the presence of thin-walled, distended and fragile microvessels as well as some thin cords of endothelial cells (without lumens or basement membranes). Disturbed molecular mechanisms that may contribute to unpredictable tissue and vessel breakdown include matrix metalloproteinases and their tissue inhibitors.²⁵⁻³⁰

This was a relatively small study for assessing differences in bleeding patterns between Cyclofem and DMPA in the chosen population and therefore only limited conclusions can be drawn. The numbers of bleeding and spotting days per 90-day reference period were fairly similar between DMPA and Cyclofem users in this study, but this simple analysis was not specifically aimed at demonstrating differences in the detailed patterns of experience in individual women. Research elsewhere has demonstrated that Cyclofem users do tend to have more regular and predictable patterns of bleeding, but that some irregularities of light bleeding and spotting, and of amenorrhoea, are not uncommon.⁴⁻⁶ These bleeding irregularities are a leading reason for premature discontinuation of use of both of these injectables,^{4-6,31-33} a problem that was also seen in the present study. Iranian women also expressed a concern about the inconvenience of the frequency of the monthly injections of Cyclofem, and a few dropped out because of this.

Figure 5 Mean $\pm$ SEM bleeding and spotting days in the first and second 3-month time intervals of injectable contraceptive use. DMPA, depot medroxyprogesterone acetate

This study has highlighted some of the major difficulties of undertaking such research in new centres within the developing world. The clinical trial structure had to be set up from scratch in two centres, including a successful clinical family planning centre, with attention to good clinical research practice. The laboratory science had to be developed within two existing pathology departments, and attention paid to close co-ordination and quality review of all data. The study participants came from a culture that was unfamiliar with clinical trials and this influenced follow-up in spite of excellent direct contact from research staff. Although the clinical staff responsible for collecting endometrial biopsies were experienced in such biopsies for infertility, the volumes of tissue collected from potentially thin and "atrophic" endometrium in Cyclofem and DMPA users was often insufficient for immunohistochemical assessment. Nevertheless, sufficient numbers of good biopsies were obtained to allow paired comparisons between control and treatment situations.

This study allowed the conclusion to be drawn that there are very similar microvascular changes within the thin endometrium associated with use of DMPA or Cyclofem, even though Cyclofem users are exposed to a significant cyclical degree of oestrogen.

In summary, DMPA and Cyclofem use are both associated with atrophic endometrium, decreased endometrial vascular density and rather similar irregular bleeding patterns, mostly with spotting and very light bleeding. Therefore, it does not appear that the addition of a small dose of oestradiol to the progestogen (in Cyclofem) has substantially influenced the endometrial vessels and bleeding patterns compared with DMPA use alone.

Acknowledgements

The authors thank The National Research Center for Reproductive Health, the National Medical Science Research, Deputy Minister of Research and Technology, Ministry of Health of Iran for their sponsorship of the project, especially Professor Hosein Malek Afzali, Deputy Minister of Research. The help with microscopy and photography given by Dr Mohamad Reza Sadeghi and Mr Ali Ahmad Ali Biat from Avesina Fertility and Infertility Research Center is acknowledged, as is the assistance with statistical analysis provided by Dr Hamid Alavi Majd.

Statements on funding and competing interests

Funding None identified.

Competing interests None identified.

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