

## Effects of combined oral contraceptives on the skin of mice

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**Aim:** To find the effects of a combination of 2 widely used contraceptives, ethinyl estradiol and cyproterone acetate (ECPA), on skin and sebaceous gland morphology and dermal vascularity of mice.

**Materials and methods:** Female mice were divided into groups: treatment with ECPA for 1 month (T) and its control (C); and cessation of treatment for an additional month (recovery, R) and its control (CR). Body weight, skin histology and morphometry, and vaginal smears were performed and analyzed.

**Results:** In the T and R groups, there was a significant increase in epidermal and dermal thickness and dermal vascularity. In group T, the number and size of sebaceous glands (SGs) decreased. In group R, all features of SGs were similar to those of group C. Estrous cycle was arrested at the diestrus stage in group T, but regained its regular pattern in group R.

**Conclusion:** A decrease in the number and size of SGs explains the mechanism through which ECPA acts. The increase in skin thickness and vascularity explains the enhancement of shape and texture induced by these hormones. The contraceptive effects of ECPA are confirmed. Changes are reversible after cessation of treatment.

**Key words:** Combined oral contraceptives, ethinyl estradiol, cyproterone acetate, skin histology

### Introduction

Hormonal therapy constitutes one of the treatment options available to women with acne. The goal of the therapy is to oppose the effects of androgens on the sebaceous gland, which in turn reduces sebum production and potentially leads to an improvement in acne. Hormonal treatment is an important option for women who are unresponsive to standard therapies and it is usually administered as an adjuvant or an alternative to those with acne of mild to moderate severity who also require contraception (1). Furthermore, certain women may be more suited to hormonal therapy than others, including those with adult-onset acne, premenstrual flare-up, irregular menses, or seborrhea (2). These agents suppress gonadotropin release, which results in the suppression of ovarian and adrenal androgen precursors, as well

as increasing sex hormone-binding globulin, which lowers the levels of biologically active testosterone. In addition, the antiandrogens competitively displace androgens at the receptor, thereby blocking their action (3).

Of the products that are available, ethinyl estradiol/cyproterone acetate (ECPA; Diane 35) is the most established and has well-documented efficacy in the treatment of acne. It is considered to be the gold-standard hormonal therapy for acne. As well as being effective in milder cases, ECPA is the only product that is effective in patients with more severe acne (4). Cyproterone acetate (CPA) is a progestational antiandrogen that blocks the androgen receptor. It is available as a monophasic product (Diane 35), which contains 35 µg ethinyl estradiol and 2 mg CPA. ECPA is indicated only for women

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who require antiandrogen therapy for acne, alopecia, or hirsutism. Apart from its antiandrogenic effect, CPA also has a pronounced progestational action. Because of its combination with ethinyl estradiol, it has the properties of a combined oral contraceptive. It is widely used in many countries (3).

Several studies have shown that biochemical and histometric parameters are suitable to demonstrate effects of drugs on epidermal metabolism and histology (5). Little is known of the effects of hormonal contraceptive agents on skin homeostasis and sebaceous gland functions. This study examines the effects of 2 widely used contraceptive agents, ethinyl estradiol and CPA, on the skin, specifically on the sebaceous glands.

## Materials and methods

### Animals

In this experiment, 20 adult female albino mice (*Mus musculus*) were used. They were obtained from the National Center for Fertility Research, Baghdad, Iraq. The animals were used according to the general guidelines of laboratory animals (Iraqi general health law, experimental protocol section, 1981) and according to the protocol of the laboratory animal center of the University of Baghdad, Iraq. The experimental protocol was approved by the College of Medicine, Al Mustansyriyah University, Baghdad, Iraq, number 6391, on 20 October 2005. The animals' ages ranged between 2 and 3 months, and their body weight range was 16–20 g at the beginning of the experiment.

The animals were divided into 4 groups of 5 mice each:

- Treated group (T): With an average body weight of 16.6 g at the start of the experiment. They received daily doses of CPA and ethinyl estradiol in the form of oral suspension per os by modified feeding syringe for 1 month, and were then sacrificed.
- Control group (C): With an average body weight of 18.7 g at the start of the experiment. This group received tap water per os by modified feeding syringe for 1 month, and were then sacrificed.

- Recovery group (R): With an average body weight of 17.6 g at the start of the experiment. They received the same course as group T, but after the end of the first month of treatment, they were left without treatment for another month and then sacrificed.
- Control for recovery group (CR): With an average body weight of 17.3 g just before the beginning of the experiment. They received ordinary tap water as did group C, but were kept for an additional month to be sacrificed with the group R animals.

All animals were dissected under anesthesia using chloroform inhalation (5.0% v/v with 2% ethanol). With the animals under anesthesia and the heart still beating, a piece of middorsal skin of 1 cm × 1 cm was removed.

### Hormones

The following hormones were used during the experiment:

1. CPA: From Schering AG, Germany, in the form of 10-mg oral tablets. The tablets were kept in a cool, dark, dry place. The dose used in the experiment was 0.4 mg/kg mouse body weight.
2. Ethinyl estradiol: From Schering AG in the form of 2-mg oral tablets. The tablets were kept in a cool, dry place and were protected from light. The dose used in the experiment was 0.01 mg/kg mouse body weight.

Tablets of each hormone were ground by mortar and pestle, and then the powder was weighed using the Mettler H54 A.R. microbalance (Karl Kolb, Germany). After that, the powder of each hormone tablet was mixed with water to form an oral suspension that was introduced directly into the stomach or lower esophagus (gavage) of a conscious mouse (6).

In humans, the effect of ECPA on sebaceous glands needs at least 6 menstrual cycles to show a good response (7), which corresponds to 1 month in mice (5-day cycles).

Animal dosage was calculated according to the following formula (8):

$$\text{HED (mg/kg)} = \text{animal dose (mg/kg)} \times \text{animal } k_m / \text{human } K_m$$

where HED is the human equivalent dose, and  $K_m$  is the conversion factor between body weight in kilograms and body surface area in square meters ( $K_m$  values were obtained from the table in reference 8).

The body weight of animals was recorded at the beginning of the experiment, weekly thereafter, and at the end of the experiment using a Mettler E2000 measure ( $d = 0.1$  g). The mean of these weights was calculated for each group.

A vaginal smear was taken from each animal daily between 1000 and 1200 hours throughout the experiment to observe cyclic changes in the estrous cycle. A final smear was taken at time of dissection. Each smear was fixed by 95% alcohol and stained with Papanicolaou stain.

### Skin preparation

A middorsal skin patch of about  $1\text{ cm} \times 1\text{ cm}$  was removed at the time of dissection and coarse hairs were shaved before the specimen was kept in formalin, and tissues were processed for light microscopy according to the method of Bancroft and Stevens (9). From each animal, 5 different sections were chosen randomly, and then 4 random readings were taken from each section, giving a total of 20 random readings per animal.

The skin was examined under a light microscope for histological changes and 4 statistical parameters were used to determine the changes in the skin and sebaceous glands.

#### 1. Epidermal and dermal thickness

For morphometric analysis, epidermal and dermal thicknesses were measured manually with a calibrated ocular micrometer scale introduced into the microscope eyepiece. Linear measurements of the epidermis were made from the basement membrane of the stratum basale to the end of the stratum granulosum in interfollicular sites. The stratum corneum itself could not be measured, since it was partially or completely torn off during sectioning. Dermal measurements were taken from the basement membrane of the stratum basale, at the site of epidermal ridges, to the hypodermal fat layer in interfollicular regions.

#### 2. Morphometric assessments of sebaceous glands and vascularity

A point-counting method was used to assess changes in the percentage of sebaceous glands in the skin samples. A square grid ( $105 \times 105\ \mu\text{m}$ ) with 121 intersecting points was used. The number of intersection points falling on an element of interest (sebaceous glands or blood vessels) was recorded and divided by the total number of points to yield the relative percentage or area fraction of that element (10).

#### 3. Sebocyte number

Sebocyte and necrotic cell numbers per square millimeter were calculated by counting their nuclei using a Zeiss integrating micrometer disk turret (Germany) and an objective micrometer slide and lens (Japan) according to the following formula (11):

$$A_i = P_i \frac{\sqrt{3}}{2} Z^2$$

where  $A_i$  is the part of the total section area belonging to the tissue component  $i$  (sebaceous gland),  $P_i$  is the number of points falling on the nuclei of that part, and  $Z$  is the equivalent in millimeters of the distance between 2 points on the integration system.

The mean cell nucleus diameter was then calculated to determine the numerical density (i.e. cell number per cubic millimeter of target tissue) using the following formula (12):

$$N_v = N_a / (D + t),$$

where  $N_v$  is the number of cells per cubic millimeter,  $N_a$  is the number of their profile per square millimeter,  $D$  is the mean diameter of the cell nucleus, and  $t$  is the section thickness in millimeter.

#### 4. Sebocyte size

Cellular circular profiles were measured using an optical micrometer and volume diameter was calculated using Giger and Riedwyl's formula (13):

$$D = (4/\pi)d,$$

where  $D$  is the mean diameter of the cell and  $d$  is the mean diameter of its circular profiles.

### Vaginal smears and estrous cycle length determination

Stages of the estrous cycle were determined by cytological evaluation of vaginal smears. Warm saline was gently flushed into the vagina using a soft plastic pipette between 1000 and 1200 hours daily over a period spanning at least 6 consecutive cycles. The lavages were smeared onto glass slides, stained with Papanicolaou stain, and examined microscopically to evaluate the cytological features described in Results below. The average length of each phase was calculated for each individual mouse and used to calculate the average length  $\pm$  standard deviation (SD) for the entire study group.

### Analysis of data

By using analysis of variance, the results were evaluated statistically, and whenever there was a difference between correlated groups, Student's t-test was applied to estimate the degree of significance by comparing the mean of the data using the SD for each group (14). The mean  $\pm$  SD of each numeric

measurement was calculated, and then each group was compared with its control.

## Results

### Body weight

There was no significant change ( $P > 0.05$ ) in body weight acquirement between the treated animals and their controls over the period of 4 and 8 weeks (Table 1). The increase in body weight fell within the expected normal limits for the treated and recovery animals as concluded from their corresponding controls.

### Skin histology

In treated and recovery animals there were more cell layers in the epidermis as compared to their corresponding controls (Figures 1a–1c and 2a–2b). The epidermis was clearly recognized with some stratum corneal sloughing and prominent cuboidal basal cells at the stratum basale.

Table 1. Changes in body weight of female albino mice.

Group	Time / body weight (g)		
	Beginning of experiment	4 weeks	8 weeks
Treated	16.6 $\pm$ 1.7	18.6 $\pm$ 1.6	-
Control	18.7 $\pm$ 1.2	20.7 $\pm$ 0.98	-
Recovery	17.6 $\pm$ 1.3	18.9 $\pm$ 1.1	21.3 $\pm$ 0.9
Control of recovery	17.3 $\pm$ 2.1	18.7 $\pm$ 1.7	19.8 $\pm$ 1.3

Data represent mean  $\pm$  SD. No statistical significance,  $P > 0.05$ .

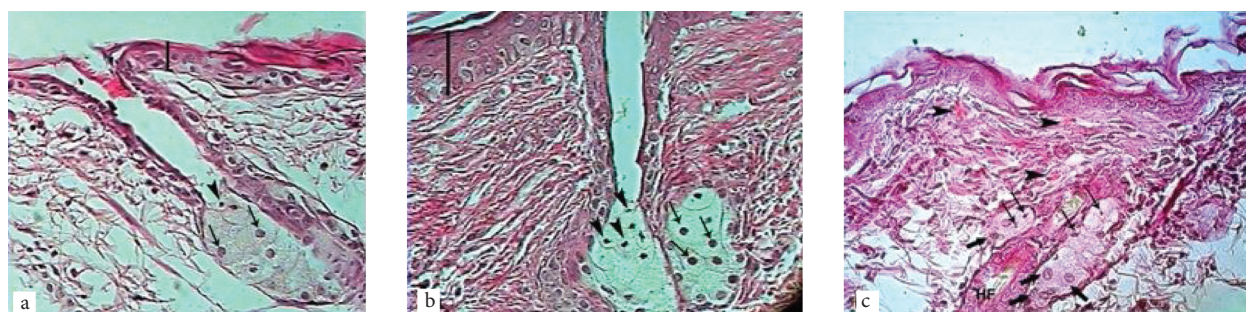


Figure 1. Skin of studied mice: a) skin of control female mouse showing thin epidermis (black bar), low dermal collagen density, light-staining sebocytic nuclei (narrow arrows), and few pyknotic nuclei (arrow head), H&E, 200 $\times$ ; b) skin of treated female mouse showing epidermal thickening (black bar), increased collagen density, darker-staining sebocytic nuclei (narrow arrows), and numerous pyknotic nuclei (arrow heads), H&E, 200 $\times$ ; c) skin of treated female mouse showing increased dermal collagen content and vascularity (arrow heads), small immature (dark-staining) sebocytes (thick arrows), and several pyknotic nuclei of sebocytes (narrow arrows) (HF = hair follicle), H&E, 200 $\times$ .



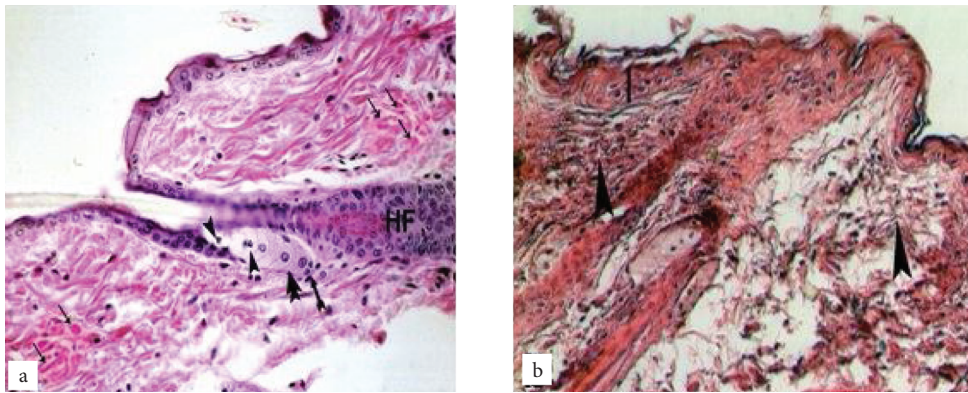


Figure 2. Skin of recovery group animals: a) skin of recovery female mouse showing dermal collagen weaving pattern, increased vascularity (narrow arrows), and small-sized sebocytes at different stages of growth with peripheral cells (tailed arrow), maturing cells (double arrow), and holocrine cells with pyknotic nuclei (arrow heads) (HF = hair follicle), H&E, 200x; b) skin of recovery animal showing increased epidermal thickness (vertical bar) and dermal dense collagenous connective tissue bundles (arrow heads), H&E, 200x.

In the dermis, dense collagenous connective tissue bundles were more pronounced in the treated and recovery animals. These collagen bundles had a basket weave pattern arrangement (Figures 1 and 2).

Morphometric measurements revealed a statistically significant increase in epidermal thickness in treated animals as compared to control animals ( $P < 0.05$ ). Epidermal thickness in recovery animals was also significantly greater than that of their control ( $P < 0.05$ ) (Table 2).

There was a highly significant increase in dermal thickness ( $P < 0.001$ ) in both treated and recovery groups as compared to their corresponding controls (Table 2).

### Skin vascularity

An increased vascularity was observed in the treated and recovery groups. Dilatation of blood vessels rather than an increase in their number was clearly obvious (Figures 1c and 2a).

There was a significant increase in blood vessel area fraction ( $P < 0.05$ ) in treated and recovery groups as compared to their corresponding controls (Table 3).

### Sebaceous glands

The sebaceous glands appeared smaller in size in treated animals as compared to the control (Figures 3a and 3b). The sebocytes of treated animals were less in number and smaller in size with darker nuclei and

Table 2. Changes in epidermal and dermal thickness in female albino mice.

Group	Epidermal thickness (µm)	Dermal thickness (µm)
Treated	22.4 ± 0.6*	315.6 ± 33.6**
Control	12.4 ± 0.4	142.6 ± 17.8
Recovery	26.6 ± 1.1*	369.6 ± 18.4**
Control of recovery	14.2 ± 2.3	217 ± 23.3

Data represent mean ± SD. \* = statistical significance,  $P < 0.05$ ; \*\* = high statistical significance,  $P < 0.001$ .

Table 3. Changes in skin vascularity.

Group	Dermal blood vessel area fraction (%)
Treated	6.3 ± 1.6*
Control	2.6 ± 0.9
Recovery	5.6 ± 1.1*
Control of recovery	2.1 ± 1.3

Data represent mean ± SD. \* = statistical significance,  $P < 0.05$ .

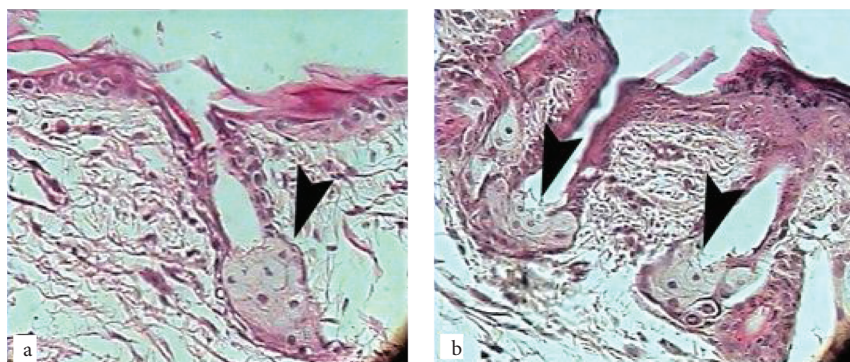


Figure 3. Sebaceous glands of studied mice: a) sebaceous gland of control female mouse showing large sebocytes and lightly staining nuclei with obviously larger glandular size (arrow head), H&E, 200 $\times$ ; b) sebaceous gland of treated female mouse showing smaller sebocytes and darkly staining sebocytic nuclei with obviously smaller glandular size (arrow heads), H&E, 200 $\times$ .

less foamy cytoplasm, and more necrotic cells with absent or pyknotic nuclei, as compared to the control animals (Figures 3a and 3b). In recovery animals, all features of sebaceous glands and their sebocytes were like those of the control animals (Figure 2a).

The sebaceous glands' area fraction was significantly reduced ( $P < 0.05$ ) in treated animals, but not in the recovery group (Table 4). The number and size of sebocytes decreased significantly ( $P < 0.05$ ) among the treated group, while the increase in the number of necrotic cells in treated animals was highly significant ( $P < 0.001$ ) compared to the control animals (Table 4).

#### Vaginal smears and estrous cycle changes

Histological examination of the smears obtained from control groups showed regular estrous cycles with a length of 4–5 days, whereas smears obtained from treated animals showed that the cycle was

arrested at the diestrus stage, starting 4–5 days from the beginning of the treatment, and remained in this stage for the whole period of treatment (Figures 4a and 4b). These treated animals' smears contained mainly leucocytes but also a variable number of epithelial and small cornified cells. The recovery group regained their regular estrous cycling pattern during the second 4 weeks of the experiment, after cessation of treatment (Figure 4c).

Table 5 shows the length of the estrous cycle and its stages in each animal group. The length of the whole cycle and the number of cycles during the treatment period was significantly reduced ( $P < 0.05$ ) in both treated and recovery groups (Table 5). However, the recovery group showed a more uniform distribution of phases over the cycle duration. In the treated group, about 78% of the cycle days were spent in diestrus, compared to 57% in the recovery group.

Table 4. Changes in sebaceous gland area fraction (%), sebocyte and necrotic cell number (per mm<sup>3</sup>), and sebocyte diameter (µm) in female albino mice.

Group	Sebaceous gland area fraction (%)	Sebocyte number (per mm <sup>3</sup> )	Sebocyte diameter (µm)	Necrotic cell number (per mm <sup>3</sup> )
Treated	17.3 ± 2.1*	220 ± 25.5*	44.1 ± 2.4 *	42.4 ± 7.4**
Control	32 ± 4.2	346 ± 14.2	83.2 ± 1.3	17.5 ± 3
Recovery	29 ± 3.7	310 ± 15.3	78.6 ± 4.1	21.4 ± 1.6
Control of recovery	33 ± 5.6	337 ± 24.2	85.3 ± 2.6	19.5 ± 2.8

Data represent mean ± SD. \* = statistical significance, P < 0.05; \*\* = high statistical significance, P < 0.001.

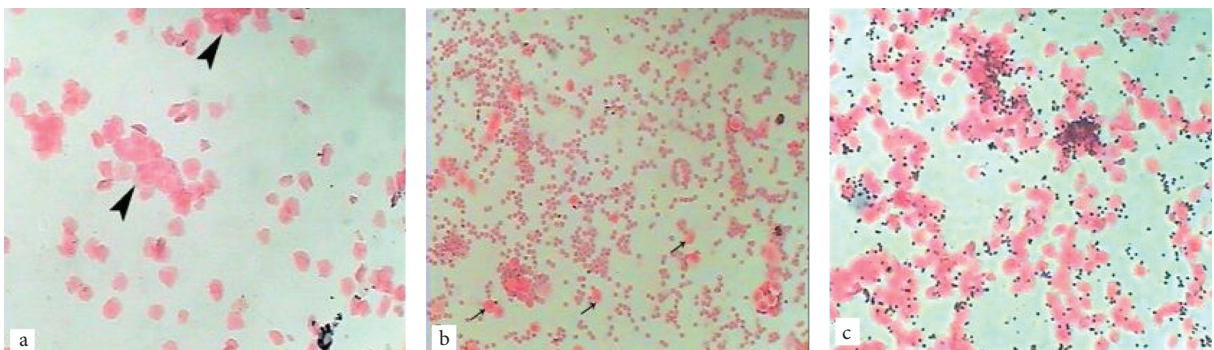


Figure 4. Vaginal smears of studied mice: a) smear of control mouse showing features of vaginal estrus with fully mature (anucleated) dark-staining cornified epithelial cells arranged in mucus clusters (arrow heads), H&E, 100×; b) smear of treated mouse showing persistent diestrus features with abundant inflammatory leukocytes (small rounded cells) with few immature (nucleated) epithelial cells (arrows), H&E, 100×; c) smear from albino mouse recovery group showing features of metestrus with dark-staining cornified and nucleated epithelial cells together with numerous inflammatory leukocytes, H&E, 100×.

Table 5. Changes in estrous cycle in female albino mice.

Group	Time (weeks)	Number of cycles per duration	Duration (days)			
			Proestrus	Estrus	Metestrus	Diestrus
Treated	4	1.3 ± 0.35 **	1.1 ± 0.1	2.3 ± 0.4	2.6 ± 0.2	22.2 ± 0.6**
Control	4	6.5 ± 0.22	4.9 ± 0.2	7.2 ± 0.2	6.3 ± 0.1	9.7 ± 0.6
Recovery	8	7.1 ± 0.36 **	6.3 ± 0.2	9.9 ± 0.1	9.2 ± 0.3	32.1 ± 0.4**
Control of recovery	8	12.8 ± 0.12	11.6 ± 0.3	16.4 ± 0.5	12.8 ± 0.3	18.8 ± 0.4

Data represent mean ± SD. \*\* = high statistical significance, P < 0.001.

## Discussion

The absence of a significant difference between the weights of treated and control animals in this study may be related to a net effect of weight loss, caused by estradiol, compensated by weight gain in the form of fluid retention caused by CPA. Estradiol causes weight loss by increasing the basal metabolic rate (15), whereas CPA causes weight gain by water retention (16).

Estrogen supplementation prevents skin from aging by increasing its thickness and improving its moisture (17) and increases collagen protein levels in aged human skin (18), whereas estrogen deprivation is associated with skin dryness, atrophy, fine wrinkling, poor healing, and declining dermal collagen content with decreased laxity (19). Thus, the increased dermis thickness of our treated animals is probably related to increased skin collagen caused by estrogen.

The epidermal thickening shown in our study may be explained by increased mitotic activity of the basal layer induced by estrogen, for estrogen is proven to stimulate proliferation of keratinocytes through estrogen alpha receptors (20).

In our study, treatment with estradiol and CPA was associated with increased dermal vascularity. Estrogens have been shown to stimulate angiogenesis through endothelial proliferation and differentiation by secretion of tissue growth factor beta-1 (21). However, angiogenesis in adult organisms was shown to be absent under normal conditions, except in the ovaries (22). Therefore, it is best to say that the increased vascularity in our treated animals was through vasodilatation rather than new vessel formation (angiogenesis). Such vasodilatation may have been caused by increased collagen synthesis and extracellular matrix secretion, requiring a bigger blood supply and more nutrient delivery.

Combined oral contraceptives have been successfully used in the treatment of acne, seborrhea, and alopecia. Estrogens reduce sebum secretion in both humans and animals by reducing lipogenesis without affecting the rate of sebocyte cellular division (23). A possible mechanism for this effect is the reduction of enzymes required for fatty acid synthesis and metabolism induced by estrogens (24).

Another mechanism may involve the antagonizing effect of estrogens to androgenic activity: rats given testosterone and estrogen simultaneously have a higher rate of mitosis but a reduction in sebaceous gland size and sebum secretion (25).

CPA significantly reduces sebaceous cell proliferation rather than lipogenesis with consequent reduction in sebum excretion rate, and these effects are mediated by the antiandrogenic properties of CPA (26).

In our study, the reduction of sebaceous gland area fraction and sebocyte size and number suggests a greater role of estradiol on sebocytic cell lipogenesis resulting in shrinkage of sebocytes and glandular surface area, together with the effect of CPA on the mitotic activity of basal sebocytes and sebocyte differentiation, which leads to a reduction in sebocyte number. The absence of a statistically significant difference in the size and number of the sebocytes in the recovery group indicates a reversible effect of estradiol and CPA.

Androgen is essential for sebaceous gland function and sebocyte proliferation. It is a major stimulus of sebaceous gland development, sebocyte differentiation, and sebum secretion (23,27). The antiandrogenic effect of CPA may have contributed to the increase in necrotic cells shown in our study. Antagonization of androgens by CPA led to the failure of sebocytes to develop into fully mature holocrine cells, which triggered a cascade of nuclear pyknotic or cellular apoptotic pathways that led to cell death.

Reduction of the estrous cycles and the persistence of the vaginal epithelium in the diestrus phase in our treated animals confirmed the contraceptive actions of estradiol and CPA. Cyclic changes of the vaginal epithelium can be seen in vaginal smears, which gives an indication of ovarian activity (28,29).

In light of the results of the present study, we can conclude that:

- The combined effects of CPA and ethinyl estradiol result in counterbalanced changes in body weight.
- The statistical analysis of morphometric and histological findings indicate that CPA and ethinyl estradiol significantly reduce sebocyte number and size, which results in a decrease in sebaceous



gland size, and this explains the implication of these hormones in the treatment of acne.

- The increase in thickness of the epidermis and dermis and the increase in skin vascularity explain the enhancement of shape and texture of skin induced by these hormones.

- Our results confirm the contraceptive effects of the above-mentioned hormones as evidenced by vaginal smears.
- Most of the histological and morphometric changes that occur during the period of treatment are reversible.

## References

1. Madden WS, Landells ID, Poulin Y, Searles GE, Smith KC, Tan JK et al. Treatment of acne vulgaris and prevention of acne scarring: Canadian consensus guidelines. *J Cutan Med Surg* 2000; 4: S2–13.
2. Shaw JC. Hormonal therapy in dermatology. *Dermatol Clin* 2001; 19: 169–78.
3. del Marmol J, Teichmann A, Gertsen K. The role of combined oral contraceptives in the management of acne and seborrhea. *Eur J Contracept Reprod Health Care* 2004; 9: 107–24.
4. Fugère P, Percival-Smith RK, Lussier-Cacan S, Davignon J, Farquhar D. Cyproterone acetate/ethinyl estradiol in the treatment of acne. A comparative dose-response study of the estrogen component. *Contraception* 1990; 42: 225–34.
5. Kietzman M, Lubach D, Heeren HJ. The mouse epidermis as a model in skin pharmacology: influences of age and sex on epidermal metabolic reactions and their circadian rhythm. *J Lab Animals* 1990; 24: 321–7.
6. Fox GJ, Barthold SW, Darrison MT, Quimby FW, Smith AI. The mouse in biomedical research. 2nd ed. New York: Academic Press; 2007. p.87–112.
7. Carlborg L. Cyproterone acetate versus levonorgestrel combined with ethinyl estradiol in the treatment of acne: results of a multicenter study. *Acta Obstet Gynecol Scand* 1986; 134: 29–32.
8. Reagan-Shaw S, Nihal M, Ahmed N. Dose translation from animal to human studies revisited. *FASEB J* 2007; 22: 659–61.
9. Bancroft JD, Stevens A. Theory and practice of histological techniques. 2nd ed. Edinburgh: Churchill Livingstone; 1982. p.482–502.
10. Tapan KB, Thomas RJ. Histomorphologic changes in aging skin: observations in the CBA mouse model. *Arch Facial Plast Surg* 2004; 6: 21–5.
11. Weibel ER, Kistler GS, Scherle WR. Practical stereological methods for morphometric cytology. *J Cell Biol* 1966; 30: 23–38.
12. Sterio DC. The unbiased estimation of the number and sizes of arbitrary particles using the disector. *J Microscopy* 1984; 134: 127–36.
13. Giger H, Riedwyl H. Stereological principles for morphometry in electron microscopic cytology. *Int Rev Cytol* 1969; 26: 260–6.
14. Daniel WW. Biostatistics: a foundation for analysis in the health sciences. 4th ed. Hoboken (NJ): Wiley; 1987. p.273–95.
15. Edelman A, Jensen J, Bulechowsky M, Cameron J. Combined oral contraceptives and body weight: do oral contraceptives cause weight gain? A primate model. *J Hum Reprod* 2010; 4: 60–2.
16. de Voogt H, Smith P, Pavone-Macaluso M, de Pauw M, Suci S. Cardiovascular side effects of diethylstilbestrol, cyproteroneacetate, medroxyprogesterone acetate and estramustine phosphate used for the treatment of advanced prostatic cancer. *J Urol* 1986; 2: 303–7.
17. Verdier-Sevrain S. Effects of estrogens on skin aging and the potential role of selective estrogen receptor modulators. *Climacteric* 2007; 10: 289–97.
18. Rittie L, Kang S, Voorhees J, Fischer G. Induction of collagen by estradiol: differences between sun-protected and photodamaged human skin in vivo. *Arch Dermatol* 2008; 144: 1129–40.
19. Hall G, Phillips T. Estrogen and skin: the effects of estrogen, menopause and hormone replacement therapy on the skin. *J Am Acad Derm* 2005; 53: 555–68.
20. Moverare S, Lindberg M, Faergemann J, Gustafsson J, Ohlsson C. Estrogen receptor alpha, but not estrogen receptor beta, is involved in the regulation of the hair follicle cycling as well as the thickness of epidermis in male mice. *J Invest Dermatol* 2002; 119: 1053–8.
21. Soares R, Guo S, Gartner F, Schmitt F, Russo J. 17 beta-estradiol-mediated vessel assembly and stabilization in tumor angiogenesis requires TGF beta and EGFR crosstalk. *Angiogenesis* 2003; 6: 271–81.
22. Fraser H. Regulation of the ovarian follicular vasculature. *Reprod Biol Endocrinol* 2006; 12: 4–18.
23. Guy R, Ridden C, Kealey T. The improved organ maintenance of the human sebaceous gland: modeling in vitro the effects of epidermal growth factor, androgens, estrogens, 13-cis retinoic acid, and phenol red. *J Invest Dermatol* 1996; 106: 454–60.

24. Lundholm L, Zang H, Hirschberg A, Gustafsson A, Arner B, Wright K. Key lipogenic gene expression can be decreased by estrogen in human adipose tissue. *Fert Steril* 2008; 90: 44–8.
25. Smith KR, Thiboutot DM. Thematic review series: skin lipids. Sebaceous gland lipids: friend or foe? *J Lipid Res* 2008; 49: 271–81.
26. Burton J, Laschet U, Shuster S. Reduction of sebum excretion in man by the antiandrogen, cyproterone acetate. *Brit J Dermatol* 2006; 89: 487–90.
27. Miyake K, Ciletti N, Liao S, Rosenfield R. Androgen receptor expression in the preputial gland and its sebocytes. *J Invest Dermatol* 1994; 103: 721–5.
28. Rao MP, Kaliwal BB. Monocrotophos induced dysfunction on estrous cycle and follicular development in mice. *J Industrial Health* 2002; 40: 237–44.
29. Ohta Y, Iguchi T. Effects of progesterone plus estradiol on vaginal epithelium showing estrogen-independent proliferation and cornification in neonatally estrogenized and androgenized mice. *Proc Jpn Acad* 1997; 52: 583–6.